# Bioprospecting Norwegian Microalgae

*Allelopathic compounds and their possible use in new future medicines*

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# Abstract

Bioprospecting is the process of discovery and commercialisation of new products from natural resources.Today the development of new products by pharmaceutical and bioindustries focuses on synthetic approaches; natural compounds are still the main source of biomolecules with genuinely novel structural features and properties. More than 15,000 natural products have been discovered by scientists studying marine algae, microbes, and invertebrates.Microalgae have adapted to compete for resources by a number of means. They can produce and excrete secondary metabolites which positively or negatively influence the growth, survival, and reproduction of predators, prey or competitors. This process is known as allelopathy and microalgae that produce allelochemicals may be a viable source of new future drugs in bioprospecting. The main aims of this Masters thesis was to investigate if phytoplankton from Norwegian waters produce secondary metabolites that could be of use in medicine and if these substances have an effect on other algae. Secondary aims are to discover if different fractionated extracts vary in their effect and if nutrient conditions during culturing may have an influence on the bioactivity of these extracts. High throughput screening of algal extracts was carried out using two bioassays. The first a viability assay to test the inhibition of growth and cytotoxicity of algal extracts on Jurkat cancer cells. The second is an apoptosis assay designed to investigate the the ability of algal extracts to cause apoptosis, again in Jurkat cells. Haptophyte *Prymnesium polylepis* was shown to have good potential in the bioassays and was chosen for nutrient limited culturing and allelopathy experiments. These experiments indicated that nutrient limitation can cause variability in the degree of growth inhibition and apoptotic activity of *Prymnesium polylepis*. Allelopathy of *P. polylepis* on the chain forming diatom *Skeletonema pseudocostatum* also varied according to growth conditions. Overall, there is a potential for the discovery of new future drugs from Norwegian microalgae and this study aims to give insight into which species and methods could be used to achieve this potential.

# Acknowledgements

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Shane Walker Blinden, June 2015.

# Contents

# Introduction

## 1.1 Bioprospecting

Bioprospecting is the process of discovery and commercialisation of new products from natural resources. Man has always used wildlife to his own end and today as many as two thirds of commercial pharmaceuticals have their roots in nature (Cragg, Grothaus, and Newman 2009). About 60% of the world'ÌĄs population relies almost entirely on plants for medication (Farnsworth 1994). Since more than 70% of the earth's surface is covered by water and most of this is contained in the oceans, it is surprising how marine bioprospecting is not as well known as its terrestrial counterpart. The sea provides a barrier to exploration that is harder to overcome than obstacles experienced on terra firma. Nevertheless, even early examples do exist. The Chinese are credited with the first use of cyanobacteria *Nostoc* during times of famine over 2000 years ago Jensen, Ginsberg, and Drapeau 2001. *Arthrospira* (Spirulina) and *Aphanizomenon* species have also a history of being used as a food source in times of need. The first large-scale use of microalgae in a commercial setting began more than 60 years ago in Asia (Borowitzka 1999). The majority of effort was put into growing algal biomass as a food source but some investigation also occurred into secondary metabolites with antimicrobial, antiviral, and anticancer activity as well as those that affected processes and pathways in the cell, aiding to the understanding of cell function (Borowitzka 1995).

Today the development of new products by pharmaceutical and bioindustries focuses on synthetic approaches; natural compounds are still the main source of biomolecules with genuinely novel structural features and properties (Harvey et al. 2010). More than 15,000 natural products have been discovered by scientists studying marine algae, microbes, and invertebrates (Salomon, Magarvey, and Sherman 2004). Whereas before bioprospecting focused on terrestrial sources, the rate of discovery of new metabolites from land based sources is decreasing and new leads are needed. Also, as infectious diseases evolve and develop resistance to existing pharmaceuticals, the marine environment can provide new means to treat fungal, parasitic, bacterial and viral disease. With 34 out of 36 phyla of

life present, the oceans harbour the greatest biodiversity on earth (Donia and Hamann 2003). It is no surprise that research into algae and marine animals such as ascidians, bryozoans, molluscs, soft corals, and sponges has shown that the marine environment can yield unique secondary metabolites not yet found terrestrially (Faulkner 1996; Attaway and Zaborsky 2013).

Chlorophyte alga *Dunaliella salina* is cultured for Îš-carotene, which has a multitude of uses including vitamin supplements, antioxidants, and a source of pigmentation for farmed prawns (Borowitzka 1999). *D. salina* can be made to overproduce and store Îš-carotene under stressful conditions such as high light intensity or nutrient starvation (Emeish 2012). Îš-carotene from *Dunaliella* is now being produced on a commercial scale in Australia, the USA and Israel. Secondary metabolites isolated from marine cyanobacteria found to target tubulin and actin filaments in eukaryotic cells (Jordan 1998) have been earmarked as potential anticancer drugs. In another study, half of the 41 cyanobacterial strains screened induced apoptosis in cancer cells (Oftedal et al. 2010). Extracts from one of these were shown to work in tandem with existing cancer drugs to kill leukemia cells without having negative effects on healthy cardiomyocytes (heart muscle cells).

Many bioprospecting undertakings have hit a wall after the discovery of a compound of interest. If the organism responsible cannot be grown in the lab, repeated excursions into the field are necessary to collect more material. Clinical trials on anticancer agent bryostatin-1 required the collection of approximately 12.6 metric tons of bryozoans to give an 18g sample (Schaufelberger et al. 1991). Approved anti-tumor agent ecteinascidin 743 had its development delayed by two decades because of difficulties in collecting enough tunicates needed to prepare the final elucidation (Molinski et al. 2009). It is for these reasons that easily cultured organisms like microalgae may be more suitable as a source for bioprospecting.

## 1.2 Allelopathy

Microalgae have adapted to compete for resources by a number of means. Changes in surface to volume ratio, production of specific enzymes, variations in nutrient requirements, luxury uptake of macronutrients and trace elements, pigment composition, photosynthetic capacity, mixotrophy, and vertical migration are all used to gain a competitive edge. Phytoplankton are also known to produce secondary metabolites that directly affect other organisms. Known as allelochemicals, these substances can positively or negatively influence the growth, survival, and reproduction of predators, prey or competitors. Allelopathy in the oceans is mediated by physical (viscosity, shear forces, low

reynolds numbers) as well as chemical (dilution) factors that have put a selective strain on the algae (Legrand et al. 2003). Allelochemicals are rapidly diluted once released into water and so need to be potent to have an effect. Due to the dynamic nature of the ocean they must work over a short time scale to be of benefit. In the lab, several secondary metabolites produced by phytoplankton have been shown to affect other algae as well as bacteria, fungi and viruses (Legrand et al. 2003). Studies in the field have also shown that allelopathy can influence population dynamics in microalgae (Keating 1977; Pratt 1966; Rojo et al. 2000). These unicellular organisms can affect access to light and nutrients, grazing, or hydrodynamics (Verity and Smetacek 1996). Toxic blooms are among the most studies allelopathic interactions due to their ecological and economical impact.

During summer 1988, a bloom of haptophyte *Prymnesium polylepis* (=*Chrysochromulina polylepis*) occurred over an area of 75,000 $km^2$ off the Scandinavian coastline. This bloom was toxic and caused widespread damage of both farmed fish and native species (Rosenberg, Lindahl, and Blanck 1988; Dahl et al. 1989; Underdal et al. 1989; Nielsen 1990; Kaas et al. 1991; Robertson 1991). *P. polylepis* was originally described as non-toxic to fish and not known to occur in the density observed in 1988 (Manton and Parke 1962). Extensive research was carried out to understand the conditions that lead to such an extraordinary event. The Norwegian Fisheries Research Council began the Harmful Algae program and research began into the effect of pH, temperature, nutrient concentration, light intensity, salinity, growth phase, and life cycle stage on toxin production in *Prymnesium polylepis* and closely related species. Out of this list, nutrient conditions seemed to be most influential factor with significantly higher hemolytic activity regardless if N or P is the limiting nutrient (N. Johansson and E. Granéli 1999). A change in pH from 8 to 9 was also found to increase toxicity of *P. polylepis* against *Heterocapsa triquetra* four fold and was deemed to be more influential than growth phase (Schmidt and Hansen 2001). It was determined that low levels of phosphorous at the end of the 1988 bloom may have contributed to its abnormal toxicity (Dahl et al. 1989; Maestrini and E Granéli 1991; Skjoldal and Dundas 1991; Edvardsen and Paasche 1998).

In 1989 another toxic bloom, this time of *Prymnesium parvum*, killed 750 metric tons of farmed salmon and trout in a fjord system in Ryfylke, W Norway (Kaartvedt et al. 1991). Blooms in subsequent years caused more economical damage but lessened in economical damage over time as fish farms closed. *P. parvum* occurs worldwide in temperate brackish waters (Edvardsen and Paasche 1998; Lundholm and Moestrup 2006) but had not caused a mass fish kill of this magnitude before in marine waters. The ichthytoxic effects in this species were assumed to be caused by prymnesium-1 and prymnesium-2, deemed highly toxic by (Igarashi Shiro|Yasumoto,Takeshi 1998). Prymnesins are harmful due to their ability to increase cell membrane permeability and disturb the balance of ions in cells. On contact with fish, these toxins can block the gill cells ability to absorb oxygen and cause

death. The wider marine community is also affected by prymnesins including; other algae (Arlstad 1991), copepods (Nejstgaard and Solberg 1996), as well as bacteria and ciliates (Fistarol, Legrand, and Edna Granéli 2003). If these microalgae, common in Norwegian waters and previously thought to be harmless, could have such a large allelopathic affect when exposed to particular conditions then there is scope for similar discoveries with other related species. It is this possibility that my project was based on.

## 1.3 Aims of this study

The algal culture collection used during this study is housed at the Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo. It contains over 230 algal strains isolated from different water masses around the world; some are more than 50 years old. Many of these are from Norwegian marine waters. These algae were the tools with which the main questions for this project would be answered. The first of these questions was which strains from the library show bioactivity with high throughput screening. Some algae selected from the library were chosen because they represented different major algal lineages. Some had shown previous toxic or allelopathic activity (like haptophytes *Prymnesium parvum* and *polylepis*) while others had not. The main aim was to screen as many strains as possible for growth inhibition and apoptosis against cancer cells. In addition, harvesting was carried out at two different time points for each strain to see if growth phase caused variation in bioactivity.

After harvesting, fractionated extraction was carried out using different solvents. This was designed to answer the second question posed in this project; which extracted fractions of the selected strains have highest bioactivity? As the initial screening was general in nature, it made sense to obtain as many fractions as possible in the hope of discovering bioactive compounds. Each extract was then tested in the bioassay to determine if the solvents used in its extraction gave a better result.

Once the first round of screening was completed and results were analysed, the next question could be addressed; how do culturing conditions (e.g. temperature, salinity, light, or nutrients) affect bioactivity. Changing environmental conditions can cause a previously benign alga to become toxic. For this study, it was decided that, based on previous research on toxic haptophytes nutrient limitation was most likely to cause a change in bioactivity. After bioactive strains were grown under nutrient limited conditions they could be used to help answer the final question; do strains have allelopathic effects on other microalgae? This can be tested in various ways such as growing donor and target algae together or cross culturing growing target algae in medium previously used to

culture the donor species, e.g. by adding filtrate from the donor culture to target cultures, which was used in this Masters thesis. This question was expanded upon by investigating if changing nutrient conditions, life cycle stage of a strain, or the method of removing live cells from donor culture preparation (filtration or lysis) could cause variability on the effect on target algae growth.

# Materials and Methods

## 2.1 High throughput screening of algae for bioactivity

The basis of this experiment was to grow Norwegian microalgae in batch cultures under good conditions (sufficient light and nutrients, suitable temperature and salinity), harvest half of the cultures during the exponential growth phase and the remainder during stationary. Solvent extractions were carried out to isolate compounds of different polarity, which were tested against Jurkat cells (human cancer cell line) to gauge a response. The objective was to see if any of the strains gave an initial response under good growth conditions so they could be chosen to continue with further experimentation. A secondary objective was to assess any variation in isolates from cultures harvested during exponential or stationary phases (i.e. as conditions become less ideal and cells may be stressed).

### 2.1.1 Culturing Algae for High Throughput Screening

Microalgae for this project were obtained from the UiO culture collection maintained at the Section for Aquatic Biology and Toxicology, Department of Biosciences, UiO Blindern. All strains used originated form Norwegian waters. Some of the species have been associated with fish kills in nature (*Prymnesium* spp. and *Karlodinium micrum* ) and are known to have allelopathic effects on other organisms, but none have been previously tested for effects on Jurkat cells. A summary s given in Table 2.1 below.

**Table 2.1:** Summary of strains included in this study. Strain code is as registered in the UiO algal culture collection of University of Oslo. Temperature was measured in degrees celsius and salinity in practical salinity units (PSU).

| Strain code | Species | Division | Origin | Salinity | Temperature |
|---|---|---|---|---|---|
| UIO 004 | *Micromonas pusilla* | Prasinophyta | Skagerrak | 25 | 16 |
| UIO 007 | *Pseudoscourfieldia marina* | Prasinophyta | Oslofjorden | 25 | 19 |
| UIO 015 | *Synecococcus sp.* | Cyanobacteria | Raunefjorden | 25 | 19 |
| UIO 018 | *Phormidium sp.* | Cyanobacteria | Oslofjorden | 25 | 16 |
| UIO 040 | *Prymnesium polylepis* | Haptophyta | Risor | 30 | 19 |
| UIO 054 | *Prymnesium parvum* | Haptophyta | Ryfylke | 30 | 19 |
| UIO 063 | *Brachiomonas submarina* | Chlorophyta | Raunefjorden | 22 | 19 |
| UIO 226 | *Dunaliella tertiolecta* | Chlorophyta | Oslofjorden | 22 | 19 |
| UIO 254 | *Karlodinium micrum* | Dinophyta | Oslofjorden | 30 | 19 |
| UIO 305 | *Becheleria cincta* | Dinophyta | Flekkefjord | 30 | 19 |
| K 0026 | *Eutreptiella braarudii* | Euglenophyta | Norway | 25 | 3 |

10mL of each strain was inoculated in cell culture flasks (50 mL, Nalgene) with 30ml IMR $^1/_2$ medium (modified version by E. Paasche of that described in Eppley et al. 1967, protocol in Appendix A). These cultures were grown in conditions as in Table 2.1 until dense enough to be used as inoculum to 1 L Erlenmeyer flasks with 900 mL IMR $^1/_2$ medium. Density was calculated before inoculation and is summarised in Table 2.2.

**Table 2.2:** Cell density of cultures before inoculation. When cell concentration could not be estimated accurately with the haemocytometer (cells clumped together, very low density), ND is used. Minimum 400 cells were counted per culture.

| Strain code | Species | Cells/mL |
|---|---|---|
| UIO 004 | *Micromonas pusilla* | ND |
| UIO 007 | *Pseudoscourfieldia marina* | 1920000 |
| UIO 015 | *Synecococcus sp.* | ND |
| UIO 018 | *Phormidium sp.* | ND |
| UIO 040 | *Prymnesium polylepis* | 112500 |
| UIO 054 | *Prymnesium parvum* | 313000 |
| UIO 063 | *Brachiomonas submarina* | 74000 |
| UIO 226 | *Dunaliella tertiolecta* | 178000 |
| UIO 254 | *Karlodinium micrum* | 34000 |
| UIO 305 | *Becheleria cincta* | 85000 |
| K 0026 | *Eutreptiella braarudii* | 2500 |

Each algal strain was cultured with 4 1L replicates, two for harvest during exponential phase and two during stationary. Cultures were then placed in a culture room of ideal temperature. Light intensity was measured using Biospherical Instruments QSL-100 and cultures were placed to give ca. 100 Îijmol photons m-2 s-1 and given a day night cycle of 14 h of light and 10 h dark. A 3mL sample from each inoculum was fixed with LugolâĂŹs solution (1%) final concentration) and initial cell concentration was determined using a light microscope and Fuchs Rosenthal haemocytometer. A minimum of 400 cells was counted, giving a confidence level of 95% with a +/- 10% margin of error.

10 mL of inoculum was added to the 1 L cultures, and equal amount to all 4 replicate cultures. Even though this lead to cultures having different starting densities, it was decided that this method was a good compromise. After 2-4 days growth (depending on strain), three 1ml samples of each replicate culture were pipetted into 48 well Falcon Tissue Culture Plates(Thermo Sceintific) and *in vivo* fluorescence was measured at 460nm with a BioTek Instruments Synergy MX plate reader. Subsequent readings were then taken at the same time of day every 48 hours (Monday, Wednesday and Friday, not on weekends). Fluorescence was used as an estimate of cell density and was graphed in excel to indicate growth phase stage. Two cultures were harvested when they reached the exponential phase and the remaining two during stationary phase. Before harvest 3ml subsamples of each culture were taken and fixed with Lugol's solution (1% final

concentration) and final cell density was determined using microscope and haemocytometer.

Cultures were harvested by vacuum filtration using 55mm Whatman GF/F glass fibre filters. Filtrate and filters were frozen at -20°C after harvesting and transported to the Department of Pharmacy, UiO for chemical extraction and fractionation.

### 2.1.2 Data analysis and graphing

Data from the plate reader was first formatted in Microsoft Excel and then exported to R for analysis and graphing. The mean, standard deviation and standard error were calculated for each culture from the three replicate measurements. Growth curves were made by plotting time (in days) against fluorescence (relative units). Max growth was calculated from the points making up the steepest part of these curves (exponential phase). K (divisions/day) is equivalent to the slope of each curve and this was calculated by linear regression. These graphs are presented later in section 3.1.1.

### 2.1.3 Chemical extractions and fractionation

A Dionex Accelereated Solvent Extraction machine (ASE 350) from Thermo Scientific was used for all extractions. Due to the large volume of material on the GF/F filters, 66mL stainless steel cells were used. A 30mm ASE extraction filter was placed in the bottom of each cell and then half filled with Dionex ASE prep diatomaceous earth. GF/F filters with harvested algal material were cut into small pieces and then placed in the cells. Each cell was topped off with diatomaceous earth, sealed and loaded in the ASE. Extraction was carried out using hexane, ethyl acetate, ethanol and water. Temperature for addition of organic solvents was set at 40°C and water at 100°C. After extraction, organic solvent fractions were left to dry by evaporation in a fume hood and those with water as a solvent were freeze-dried.

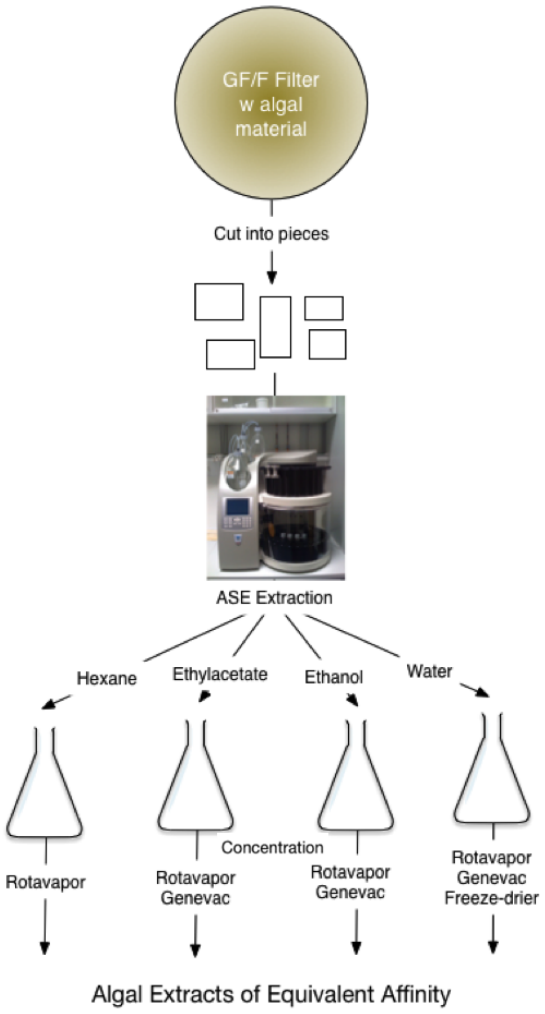


**Figure 2.1:** Flow chart for ASE chemical extraction and fractionation of algal material.

### 2.1.4 Screening for bioactivity

After extraction the fractions underwent high throughput screening at the Department of Biotechnology (person responsible Anne Jorunn Stokke). Extracts were added to 100% DMSO to obtain 10mg extract/mL DMSO and left overnight to dissolve fully.

First fractionated extracts underwent Cell Titer Glo viability assay (Promega). The DMSO-extract solution was added to 384 well Echo source plates (Labcyte) and stored at room temperature in an argon atmosphere until needed. An Echo 550 liquid handler (Labcyte) was used to transfer extracts to 384 well assay plates (Cellstar). Concentration was varied in a dilution series (100-12.5nL). Jurkat cells (LGC Standards GmbH) were seeded into assay plates (20$\mu$L, 1500 cells/well) and assays were incubated for 48 hours at 37°C (5% $CO_2$, >95% humidity). Cell viability was then detected using Cell Titer Glo kit (Promega). 20 ÂţL CTG Reagent was added to each well, plates were mixed for 1 minute on a linear shaker (Envision) and incubated in the dark for 10 minutes at room temperature. The reagent lyses cells and luminesces proportional to the amount of ATP present. An Envision 2102 multilabel reader was used to read luminescence and raw data was then transferred to Excel for analysis.

A Caspase-Glo apoptosis assay (Promega) was used to investigate apoptosis caused by the algal fractionated extracts. Extracts were added to 384 well assay plates (Cellstar) using an Echo 550 liquid handler (Labcyte). Concentration of extracts was at 50 and 100Âţg/mL. Jurkat cells were seeded into assay plates (20 Îijl, 1500 cells/well), a time=0 reading was taken and then plates were incubated for 1, 2, 6, and 24 hours respectively (37°C (5% $CO_2$, >95% humidity). 20$\mu$L Caspase Glo reagent was added to each well and plates were mixed for 30 seconds on a linear shaker. After 30 minutes incubation time luminescence was read using an Envision 2102 multilabel reader. Luminescence is proportional to the level of caspase 3/7 activity which is an essential part of apoptosis (programmed cell death). Raw data was then transferred to Excel for analysis.

## 2.2 Culture experiment with varying nutrients

Extracts of a strain of the haptophyte *Prymnesium polylepis*, strain UIO 040 showed clear activity in the bioassays and was chosen for a second round of experiments to investigate the effect of varying nutrient growth conditions on allelopathy, namely the growth of another phytoplankton species. As the UiO algal culture collection has multiple strains of *P. polylepis*, it was also decided to test both its authentic and alternate life cycle stages to see if these would have different allelopatic effects or not.

### 2.2.1 Examination in the transmission electron microscope

Whole mount electron microscopy preparations of the strains UIO 036, 037, 039 and 040 of Prymnesium polylepis were made (as described by Wenche Eikrem 1996). A droplet of each culture was placed on carbon coated copper grids, fixed with osmic acid (2% vapour) and stained with uranyl acetate (saturated solution). The grids were viewed by transmission electron microscopy (TEM) (Philips CM150 at the Electron Microscopy Laboratory for Biosciences, Department of Biosciences, University of Oslo). According to the scale types observed, it was determined that strain UIO 037 was in alternate life cycle stage and UIO 040 authentic (see Edvardsen and Paasche 1992) and so they were chosen to be part of the experiment.

### 2.2.2 Culturing algae with varying nutrient conditions

50mL cell culture flasks with 25PSU IMR $^1/_2$ algal medium (according to protocol in Appendix A) were inoculated with strains UIO 037 and UIO 040 one week prior to beginning the experiment. Inoculum cultures were then transferred to 1L Erlenmeyer flasks with 2 replicates and four nutrient conditions (8 flasks total per strain). Flasks were filled with 900mL of medium and inoculated with 10mL of inoculum culture. Light was kept at ca. 100$\mu$mol photons $m^{-2}$ $s^{-1}$ (measured with Biospherical Instruments QSL-100) and a day:night cycle (14:10 h L:D). Temperature was at a constant 19°C. Phosphate and nitrate were added to obtain the nutrient concentrations listed below.

**Table 2.3:** Nutrient conditions for each strain used in the experiment

| Treatment | Amount added ($\mu$L/L) | | Final Concentration ($\mu$M) | |
|---|---|---|---|---|
| | $PO^{4}_{3-}$ | $NO_{3-}$ | $PO^{4}_{3-}$ | $NO_{3-}$ |
| +P +N | 500 | 500 | 25 | 250 |
| -P -N | 0 | 0 | 2 | 25 |
| -P +N | 0 | 500 | 2 | 250 |
| +P -N | 500 | 0 | 25 | 25 |

Before inoculation 3mL subsamples of each culture were taken and fixed with Lugol's solution (1% final concentration). Cell density of each was determined using a haemocytometer and light microscope. The cell concentration in the inoculum was ca. 4x$10^5$ cells/mL (minimum counted cells (n)= 400).

Cell density was measured immediately after inoculation and then every two days by *in vivo* fluorescence in a plate reader (3 subsamples in 46 well plates, 460nm). Cultures were grown until a marked difference in cell density was observed for each nutrient condition (stationary phase after 8 days). Harvesting was carried out by vacuum filtration using 55mm Whatman GF/F filters. 10mL of each was transferred to cell culture flasks and kept in culture for the allelopathy stage of the experiment. The filters were frozen at -20°C until ASE extraction at Dept. Pharmacy was carried out (as in part 2.1.2). Screening for bioactivity was also carried out at Dept. of Biotechnology (as in 2.1.3).

### 2.2.3 Allelopathy experiment

10mL subsamples of each flask were taken before harvest and pooled according to replicates in 30mL glass vials (giving 8 vials total). 8mL of this was placed in an ultrasonic bath for 3 minutes to lyse cells (checked with inverted microscope to ensure no living (motile) cells remained). The remaining 12mL was preserved with Lugol's (1% final concentration) and cell density was determined with haemocytometer and microscope. 10mL filtrate from each replicate was taken during harvesting and pooled in 30ml glass vials according to replicates to give 8x 20mL samples.

A dilution series was set up after a log2 scale for each of the 16 vials. Four mL was pipetted from each vial to glass centrifuge tubes with 4mL cold 25 PSU IMR $^1/_2$ medium (50% dilution of original). This was mixed well by pipetting and 4mL was taken and added to a new tube with 4mL medium and mixed well (25% dilution). This was repeated twice more to give 12.5 and 6.25% dilutions. The resulting series was as follows 100% (original filtrate/lysed cells), 50%, 25%, 12,5%, 6.25% of original solution.

The target organism for the allelopathy experiment was *Skeletonema pseudocostatum* (strain NIVA BAC1, Bacillariophyceae). Cultures were grown in 50mL cell culture flasks at 19°C in 30mL IMR $^1/_2$ medium (34 PSU with added silicate and HCL to buffer to pH 8). These cultures were then diluted to 2000 cells/mL and 160$\mu$L was added to each well in 4x 96 well Nunclon plates and placed in experimental conditions (17°C, 100$\mu$mol photons $m^{-2}$ $s^{-1}$ light, day:night cycle of 14:10 h L:D) 24 hours before addition of the *P. polylepis* extracts. 160$\mu$L of the 5 concentrations plus a control of 100% IMR $^1/_2$ at 25 PSU were added in replicates of four to plates according to the figure below. All work was carried out in a cold sterile room at ca. 15°C to avoid unwanted effects on the *Skeletonema* due to changes in temperature.

A total of four plates were used (UIO 037 filtrate, UIO 037 lysed cells, UIO 040 filtrate, UIO 040 lysed cells). Cell density was determined using *in vivo* fluorescence and plates were placed in 17°C culture room with 100$\mu$mol photons $m^{-2}$ $s^{-1}$ light at a 14:10 L:D cycle. Fluorescence was measured at the same time daily (11am) for 7 days with the plate reader.

### 2.2.4 Analysis and statistics

Data from the plate reader was first formatted in excel and then exported to R for analysis and graphing. For the nutrient experiment mean, standard deviation and standard error were calculated for both cultures from the three replicate measurements. Growth curves were made by plotting time (days) against fluorescence (relative units). These were used to track growth during the experiment and are presented in Appendix C.

Nonlinear mixed effect modelling (nlme package, Pinheiro and Bates 2000) was used to investigate the effect of each treatment on the growth of *Skeletonema* during the allelopathy experiment. The data was grouped using the groupedData function and start points were given with nlsList and SSlogis. The data was plugged into the nlme model and efforts were made to simplify it as much as possible according to methods. Confidence interval plots were drawn to assess the variation in each factor (Asym, scal, and xmid). The ratio of standard deviation in random effects from their fixed counterparts was also calculated for the same reason. If a random factor was not varying between groups (treatments), then the model was updated to run without it. The simplified model was then tested against the original with ANOVA and AIC tests. If the AIC and p values were lower for the newer model then it was chosen for use, otherwise the more complex model was used. These steps were repeated so that Asym, scal and xmid were tested individually for each dataset until the least complex model was found for each (usually the scal effects were removed). The results form the model fit were plotted and are presented later in section 3.2.2.

# Results

## 3.1 High throughput screening for bioactivity

Eleven strains in total were screened of haptophytes, dinoflagellates, chlorophytes, cryptophytes, cyanobacteria, and euglenophytes from the UiO algal culture collection. Timing harvesting was important as the potency of extracts may change depending on growth phase stage. To determine when to harvest, cell density was measured regularly by *in vivo* fluorescence. Fluorescence (relative units) was plotted against time (days) to generate a growth curve in R. The fluorescence values measured are shown in Appwndix B.

### 3.1.1 Max growth rate curves

Once harvesting was complete, maximum growth rates were calculated for each strain where possible (minimum of three readings were needed within the exponential phase). The mean for each reading was calculated along with its standard error. Linear regression was then carried out and a line of best fit drawn. The slope of this line is equivalent to growth rate (k), the amount of divisions per day.

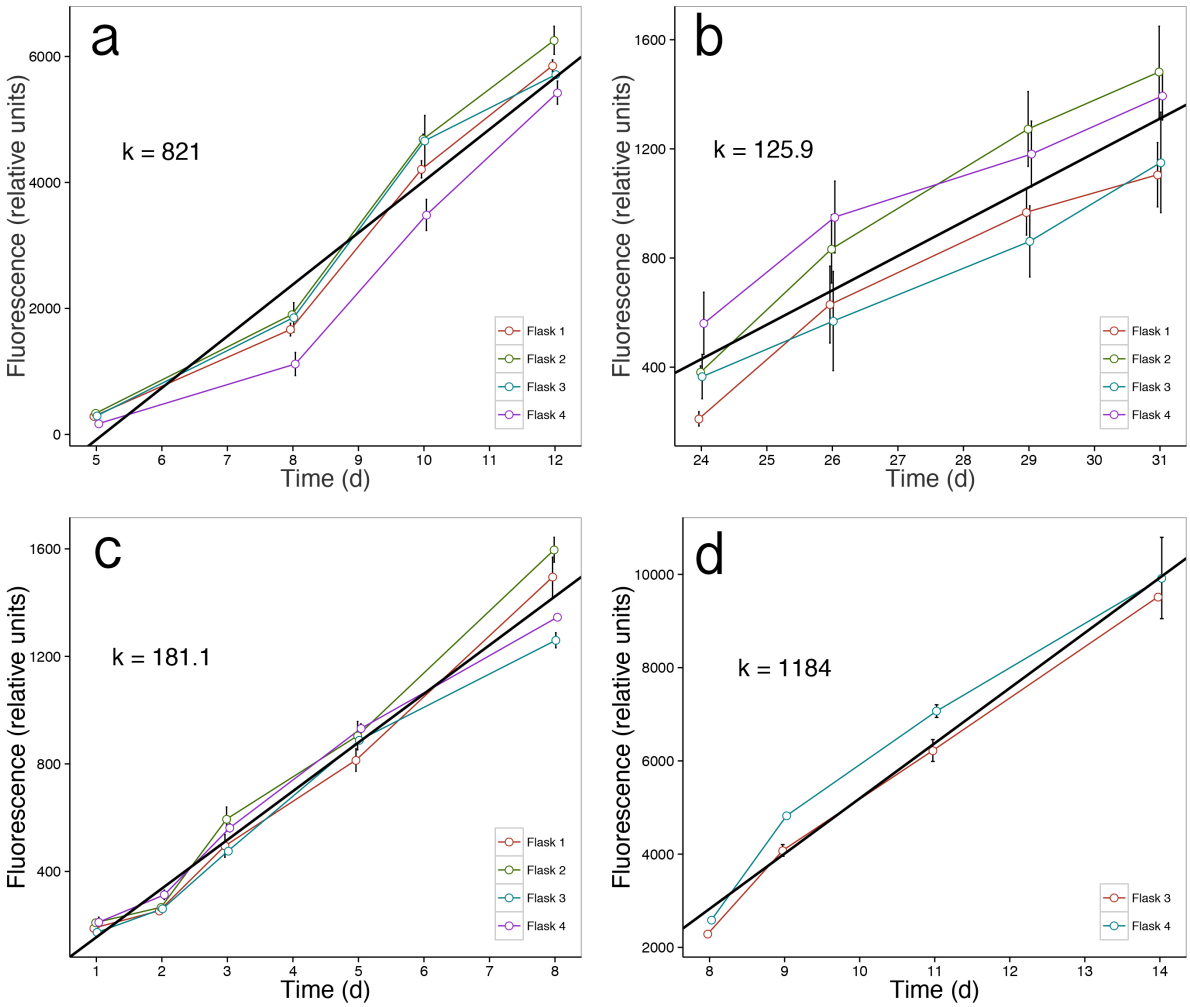


**Figure 3.1:** *In vivo* fluorescence of; a) *Pseudoscourfieldia marina*, b) *Eutreptiella braarudii*, c) *Prymnesium polylepis*, and d) *Prymnesium parvum* as measured by the plate reader. Each point is the mean of 3 subsamples, standard error is shown with black error bars. Linear regression was carried out and is shown as the black line of best fit. *Prymnesium parvum* flask one and two were harvested before the exponential phase had ended and so only data from the two stationary phase cultures are included. Note different scales on the both axes.

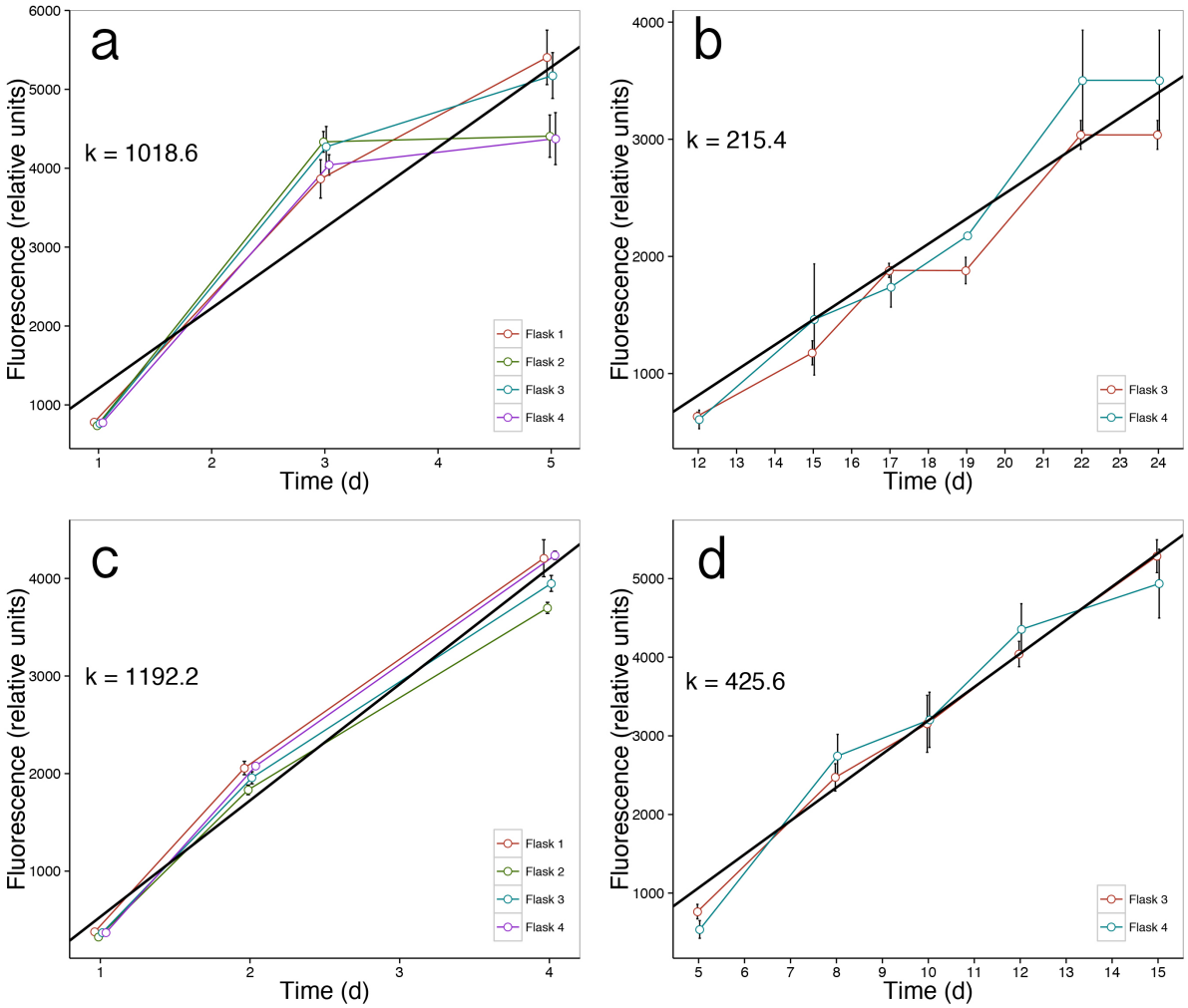


**Figure 3.2:** *In vivo* fluorescence of; a) *Brachimonas submarina*, b) *Karlodinium veneficum*, c) *Biecheleria cincta*, and d) *Dunaliella tertiolecta* as measured by the plate reader. Each point is the mean of 3 subsamples. Standard error is shown with black error bars. Linear regression was carried out and is shown as the black line of best fit. *Karlodinium veneficum* and *Dunaliella tertiolecta* exponential cultures were harvested before the exponential phase had ended and so only data from the two stationary phase cultures are included. Note different scales on the both axes.

### 3.1.2 Viability and apoptosis assays

The results from the Cell Titer Glo (viability) and Caspase Glo (apoptosis) assays are summarised in the figures below. Apoptosis assay plots show caspase 3/7 activity read as luminescence after 1, 2, 6, or 22 hours incubation at concentrations of 50 and 100$\mu$g/ml. Higher levels of caspase 3/7 activity at shorter incubation times indicate that an extract may be of interest, as too are higher responses at lower concentrations.

Viability was assessed at five different extract doses; 100, 50, 16.3, 5, and 1.25$\mu$g/ml. The log of these concentrations was plotted against the percentage of growth inhibition (ATP concentration after 48h incubation period). These curves were then fit against a 4 parameter logistic nonlinear regression model to estimate the half maximal inhibitory concentration ($IC_{50}$). If the data fit the model well, $IC_{50}$ was given based on the inflection point of the curve (C-parameter). $IC_{50}$ is measured in $\mu$g/ml and so lower values mean higher bioactivity and the extract can be considered more potent.

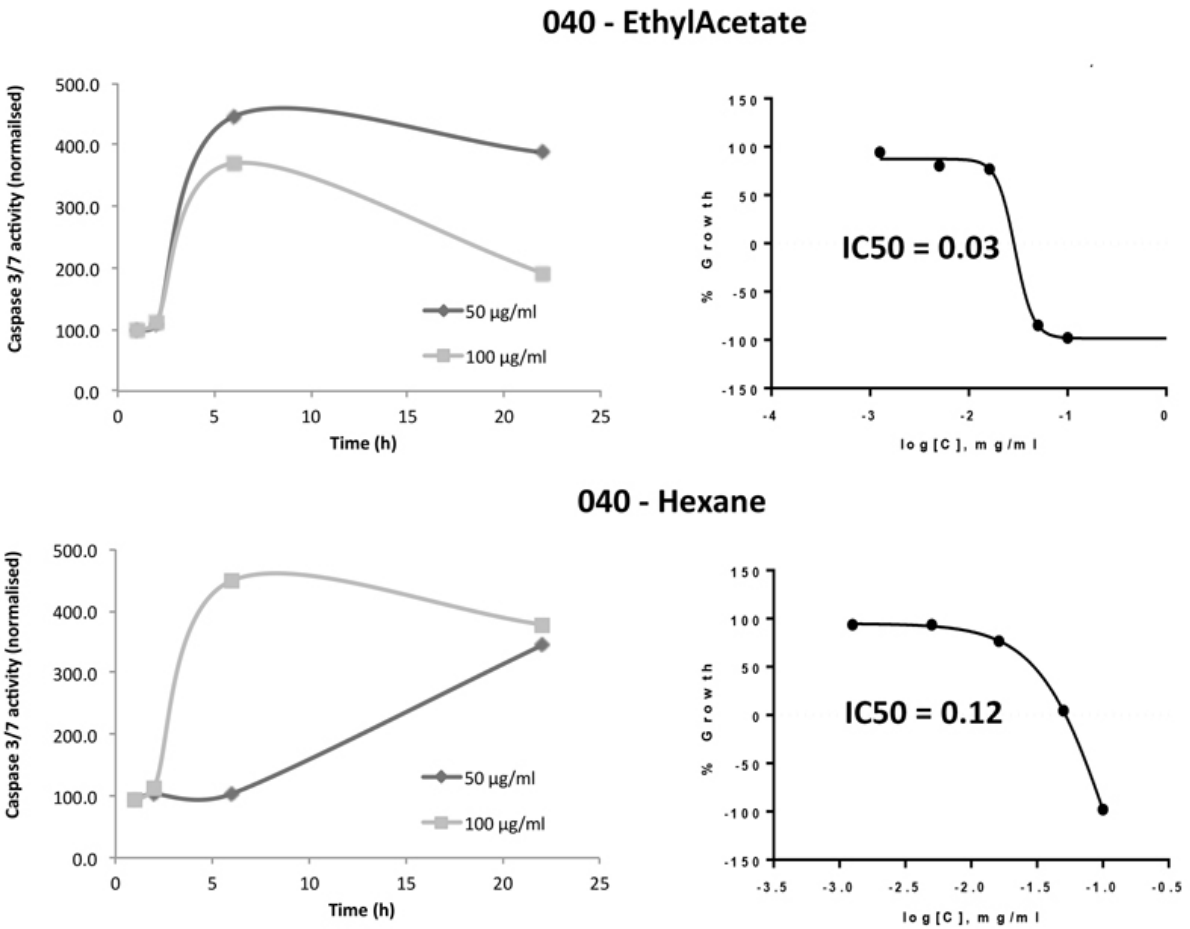


**Figure 3.3:** Assay results from UIO 040 *Prymnesium polylepis* stationary phase, ethyl acetate and hexane extractions. The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

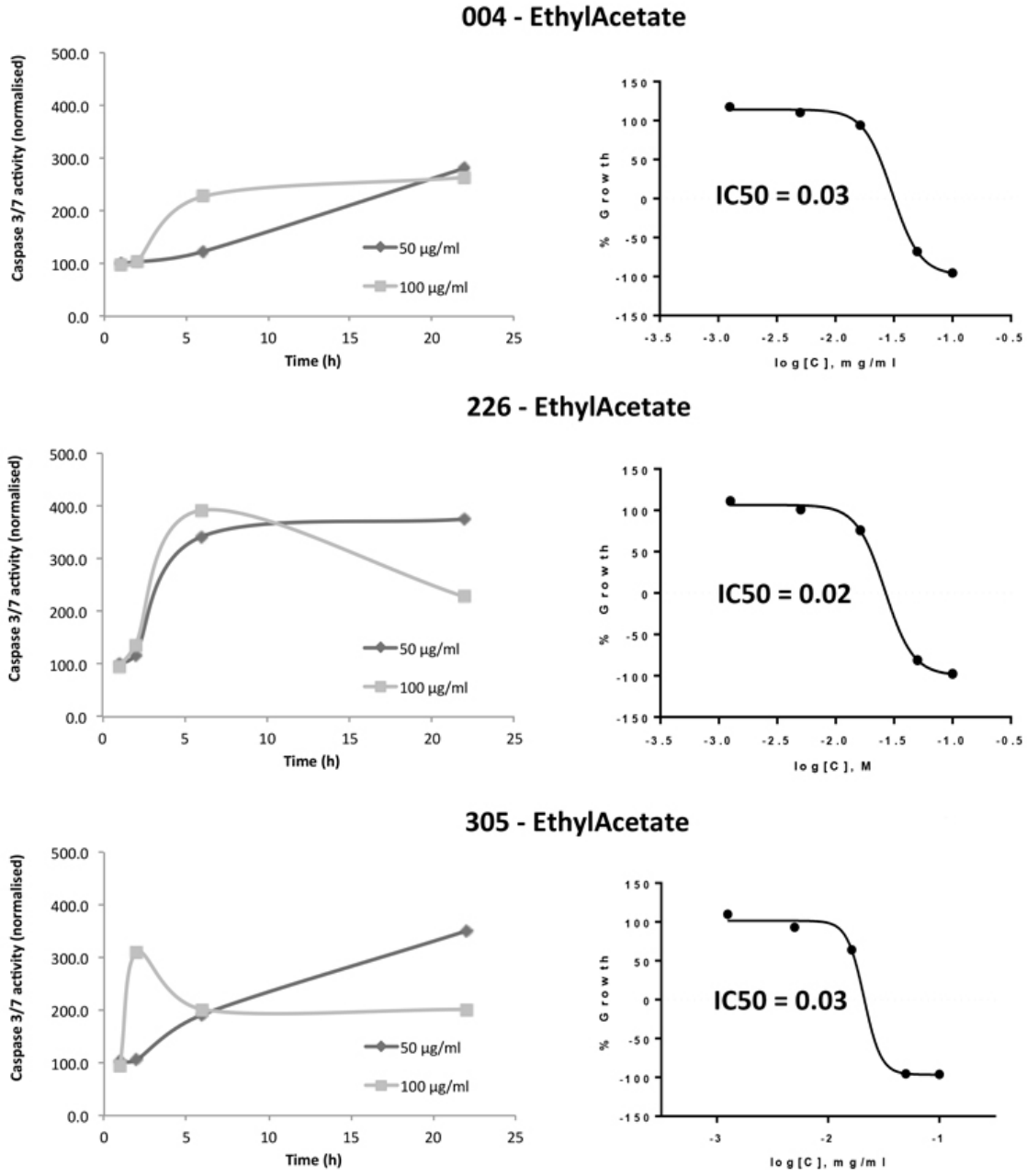


**Figure 3.4:** Assay results from UIO 004 *Micromonas pusilla* (stationary phase), UIO 226 *Dunaliella tertiolecta* (exponential phase), and UIO 305 *Biecheleria cincta* (stationary phase) ethyl acetate extractions. The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

## 3.2 Nutrient experiments

### 3.2.1 Nutrient experiment growth curves

*Prymnesium polylepis* was chosen for nutrient and allelopathy experiments based on the bioassay results. Previous research has shown that toxicity of *P. polylepis* can vary between authentic and alternate life cycle stages and so it was necessary to determine which life cycle stage each of the four strains in the UiO culture collection was in. Whole mounts of UIO 036, 037, 039, and 040 were prepared for examination by TEM and it was determined that UIO 037 was alternate and UIO 040 authentic cell type. Both of these strains were grown under four different nutrient treatments (+P+N, -P-N, -P+N, +P-N) to assess if these conditions had any effect on bioassay screenings and later allelopathy experiments.

Growth curves below were generated in R from *in vivo* fluorescence data taken by the plate reader during the nutrient limited or depleted culturing of the two *P. polylepis* strains. Due to an unexpected growth spurt, readings only began during the later stage of the exponential phase. Despite this, the plots show a clear difference between the four treatments. The cultures with full nutrient levels reached highest cell density. The cultures that obtained the next highest density were those with added nitrate, but no phosphate. It could be these cultures still managed to grow relatively well as microalgae have the ability store phosphorus or use bacteria as a source and these cultures were non-axenic Nygaard and Tobiesen 1993 . Both strains with treatments -P-N and +P-N grew to similar density, showing that nitrate limitation was more of an inhibiting factor than phosphate.

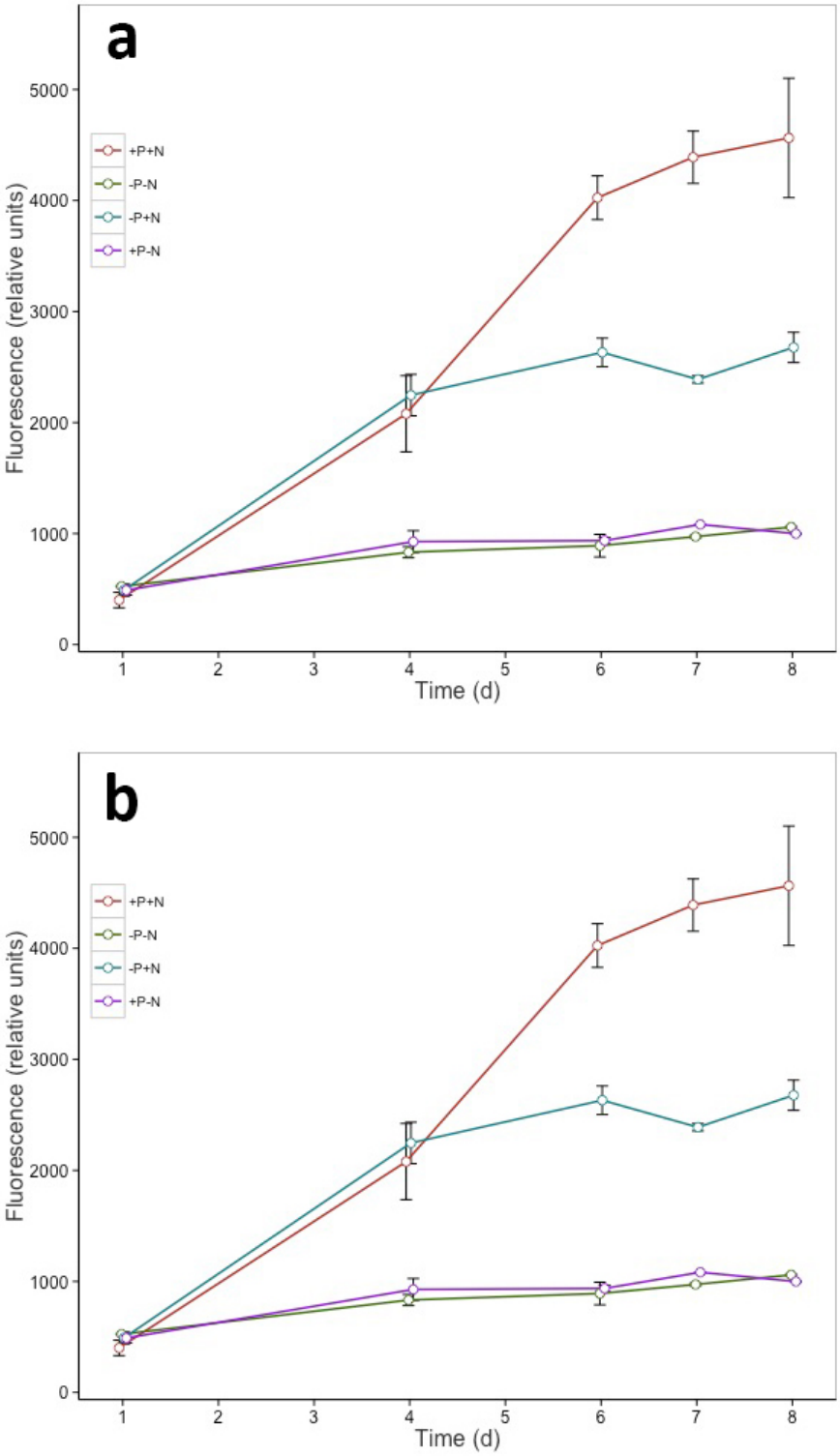


**Figure 3.5:** Plots showing *in vivo* fluorescence measurements over time illustrating the growth of a) UIO 037, and b) UIO 040 as cultured under different nutrient conditions. Each point is the mean of three sub samples and the standard error is shown in black error bars.

## 3.3 Bioassays of nutrient experiment

Apoptosis and viability assays for the nutrient experiment were carried out in much the same way as the first round. Viability was estimated by measuring ATP concentration in Jurkat cells after incubation with extracts for 48 hours. Caspase 5/7 activity was used to estimate apoptosis in Jurkat cells at 50 and 100$\mu$g/mL doses after 1, 2, 6, and 24 hours incubation time. Alternate strain UIO 037 showed more activity than UIO 040 with only one UIO 040 viability assay fitting the 4PL model well and getting an $IC_{50}$ value. Hexane extracts had a more marked effect on viability with all of the UIO 037 hexane extracts returning results.

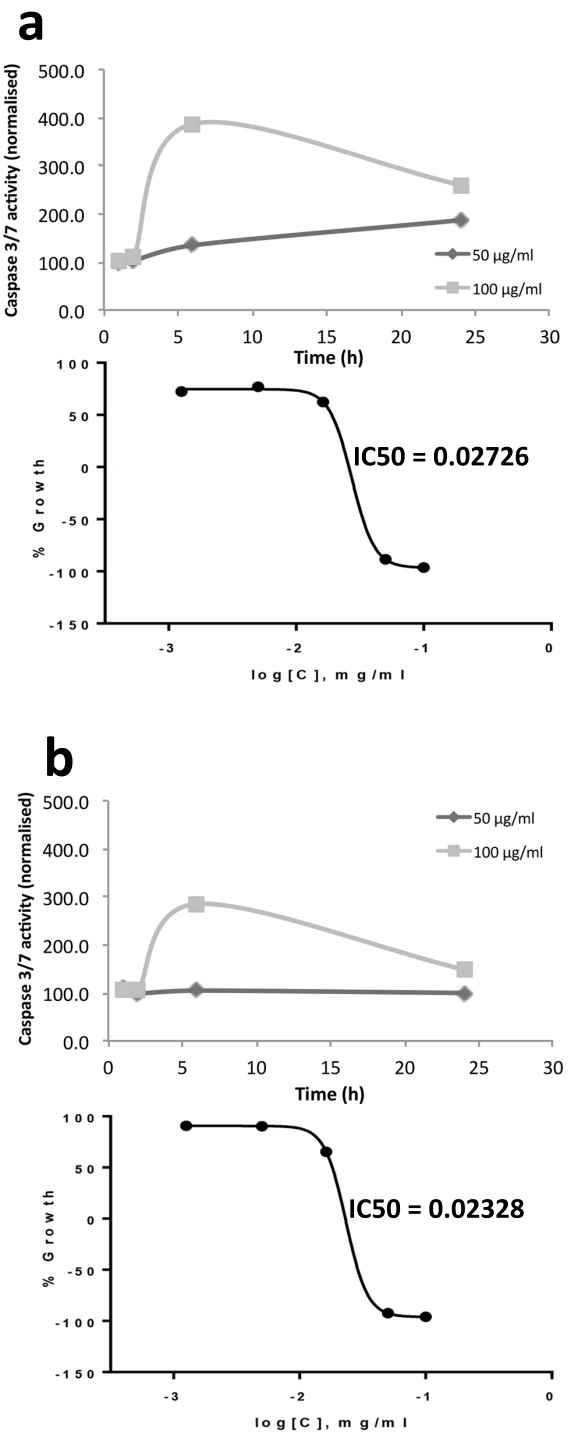


**Figure 3.6:** Assay results from UIO 037 *Prymnesium polylepis* +P+N, a) hexane and b) ethyl acetate extractions. The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

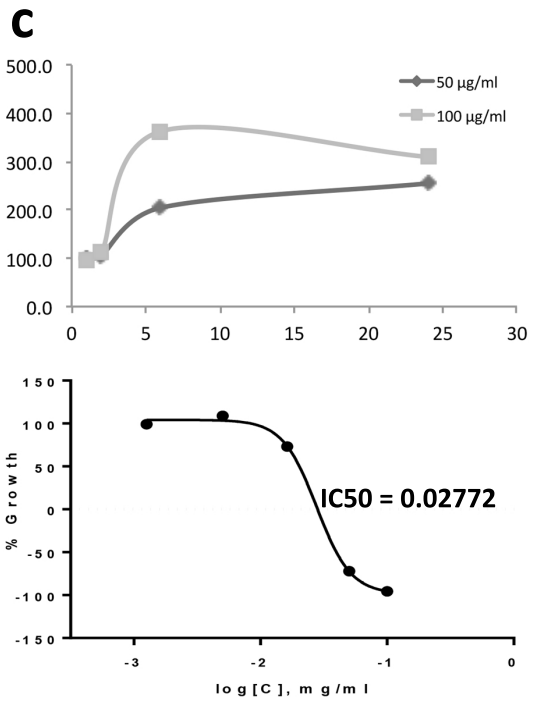


**Figure 3.7:** Assay results from UIO 037 *Prymnesium polylepis* -P-N, hexane extraction (ethyl acetate did not cause enough activity to obtain $IC_{50}$ value). The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

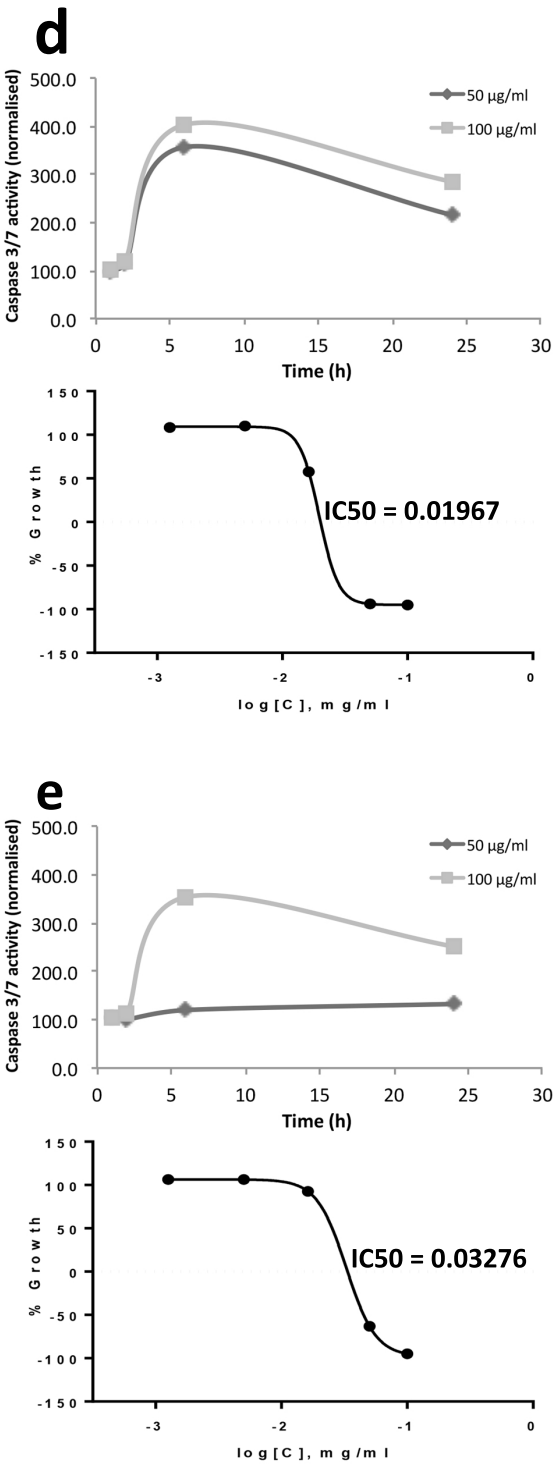


**Figure 3.8:** Assay results from UIO 037 *Prymnesium polylepis* -P+N, d) hexane and e) ethyl acetate extractions. The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

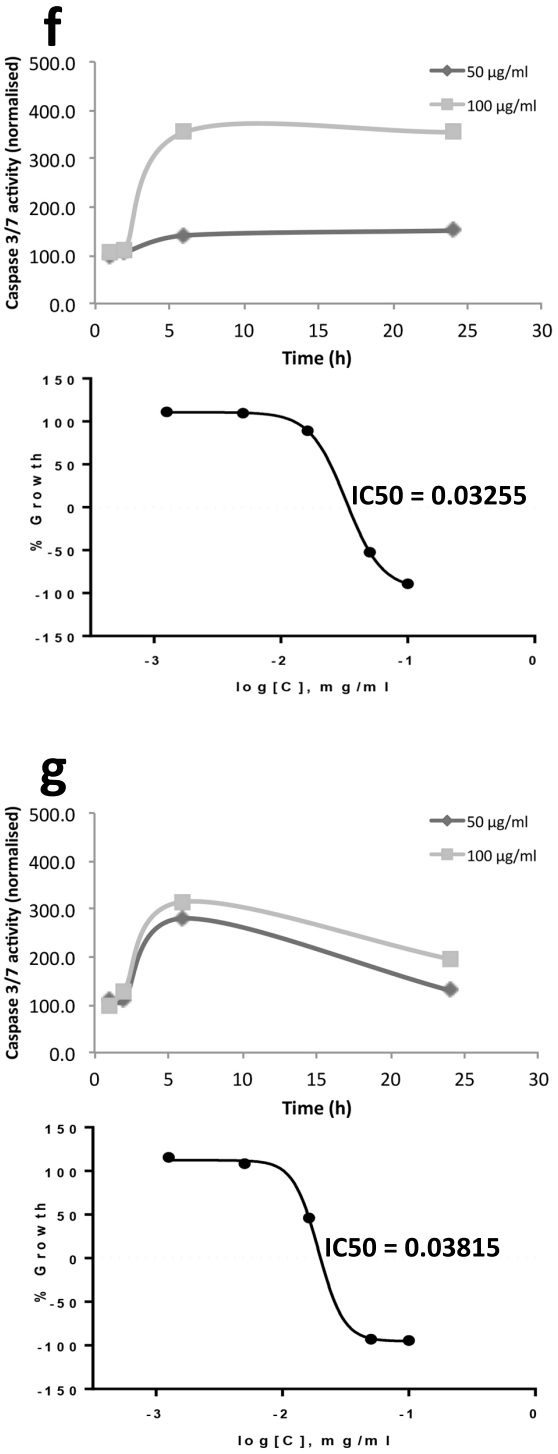


**Figure 3.9:** Assay results from UIO 037 *Prymnesium polylepis* -P-N, f) hexane and g) ethyl acetate extractions. The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

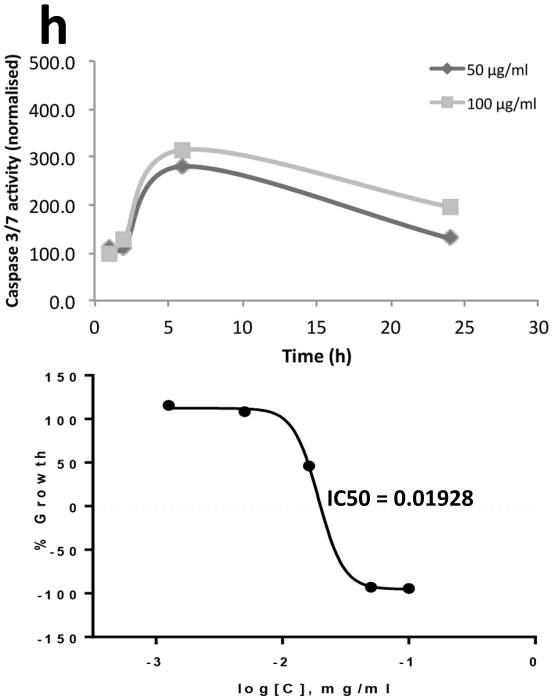


**Figure 3.10:** Assay results from UIO 040 *Prymnesium polylepis* +P-N, ethyl acetate extraction (hexane did not cause enough activity to obtain $IC_{50}$ value). The apoptosis response curve (left) shows caspase activity read for two concentrations at each time point. The viability dose response curves (right) show growth inhibition (positive % growth) and cytotoxicity (negative % growth). $IC_{50}$ is the point at which the curve crosses zero on the y-axis (mg/mL).

UIO 040 did not show enough activity in the viability assay with ethyl acetate extracts from the +P+N treatment being the only ones to fit the 4PL model and get a $IC_{50}$ value. At 0.01928$\mu$g/mL it is lower than any $IC_{50}$ values from UIO 037 in this round of tests and from any algae in the first screen (figures 3.3 and 3.4). Apoptosis for this extract was lower than in previous tests (peak of 315 now versus 370 as shown in figure 3.3) and both doses caused a similar response whereas before the 50$\mu$g/mL dose caused a higher level of cell death. Except for the -P-N ethyl acetate extraction, all nutrient treatments and extraction methods for UIO 037 caused enough variation in the viability assay to fit the model and gain $IC_{50}$ values. The lowest $IC_{50}$ was for the hexane extraction of cultures grown in -P+N conditions at 0.01967$\mu$g/mL.

Apoptosis was in general lower for UIO 040 extracts than UIO 037 (graphs in Appendix D). UIO 037 extracts form the -P+N hexane extracts caused the highest level of apoptosis in this round (403.3 after 6 hours incubation with 100$\mu$g/mL dose).

## 3.4 Allelopathy experiment

Target cells of *Skeletonema pseudocostatum* in 96 well plates were treated with six concentrations (100, 50, 25, 12.5, 6.25, 0%) of two types of extracts (filtrate or lysed cells) from two *P. polylepis* strains (UIO 037 and 040, donors) grown under 4 nutrient conditions (+P+N, -P-N, -P+N, +P-N). Each variable had four replicates and *in vivo* fluorescence readings were taken every 24 hours for seven days to track cell density of *Skeletonema*. The aim was to assess if the treatments inhibited the growth of *Skeletonema pseudocostatum* and if donor algal strain (cell type) or nutrient treatment had an effect on this. Nonlinear mixed effect modelling was used to consider the influence of random effects outside of experimental variables to judge if changes in growth were due to the applied treatments.

### 3.4.1 Nlme fit of allelopathy experiment data

In order to make compiling data and coding for each model as simple as possible, nutrient conditions that donor *Prymnesium polylepis* strains were cultured under were assigned letters a, b, c, and d (for +P+N, -P-N, -P+N, and +P-N). Concentrations of treatment were assigned numbers 1-6 in order of decreasing concentration (100, 50, 25, 12.5, 6.25, and 0%). When reading the graphs (FIGURE 8) below, window a1 corresponds to a treatment of donor cells grown in +P+N added at a concentration of 100%) to *Skeletonema pseudocostatum* target cells. The blue âĂIJfixedâĂİ line is the predicted growth curve of the target cells without inhibition and any deviation of this pink âĂIJflaskâĂİ line (actual growth) from this may be interpreted as variation caused by treatment. The height of these curves is termed the asymptote and the greater the distance between these is the easiest way to understand any variation. The inflection point of the curve on the x-axis (xmid) and the slope of the curve (scal) can also be used to determine any change due to experimental variables but AIC and ANOVA tests on these showed that they were less significant than asymptotic height.

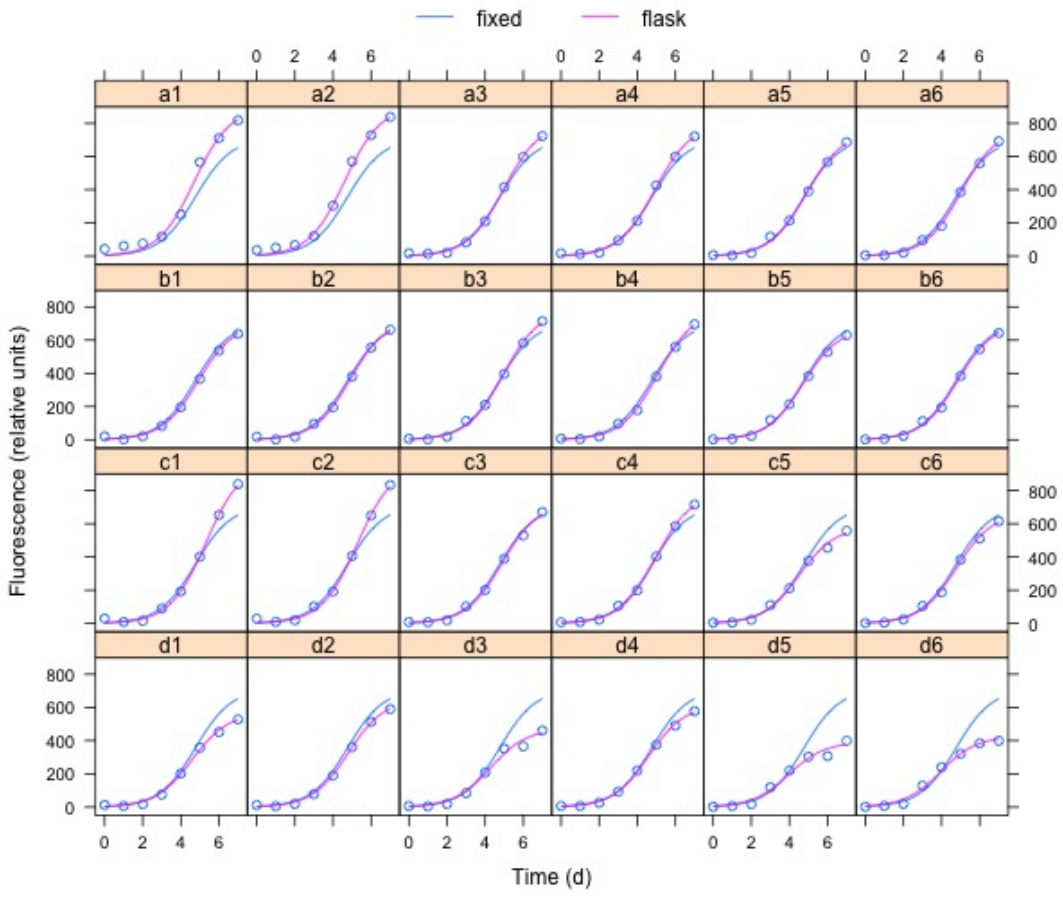


**Figure 3.11:** R plot showing fit of nlme models for *Skeletonema pseudocostatum* treated with UIO 037 lysed cells. Letters a-d in each window represent each nutrient treatment (+P+N, -P-N, -P+N, +P-N). Numbers 1-6 in each window represent concentration of treatment added (100, 50, 25, 12.5, 6.25, and 0%).

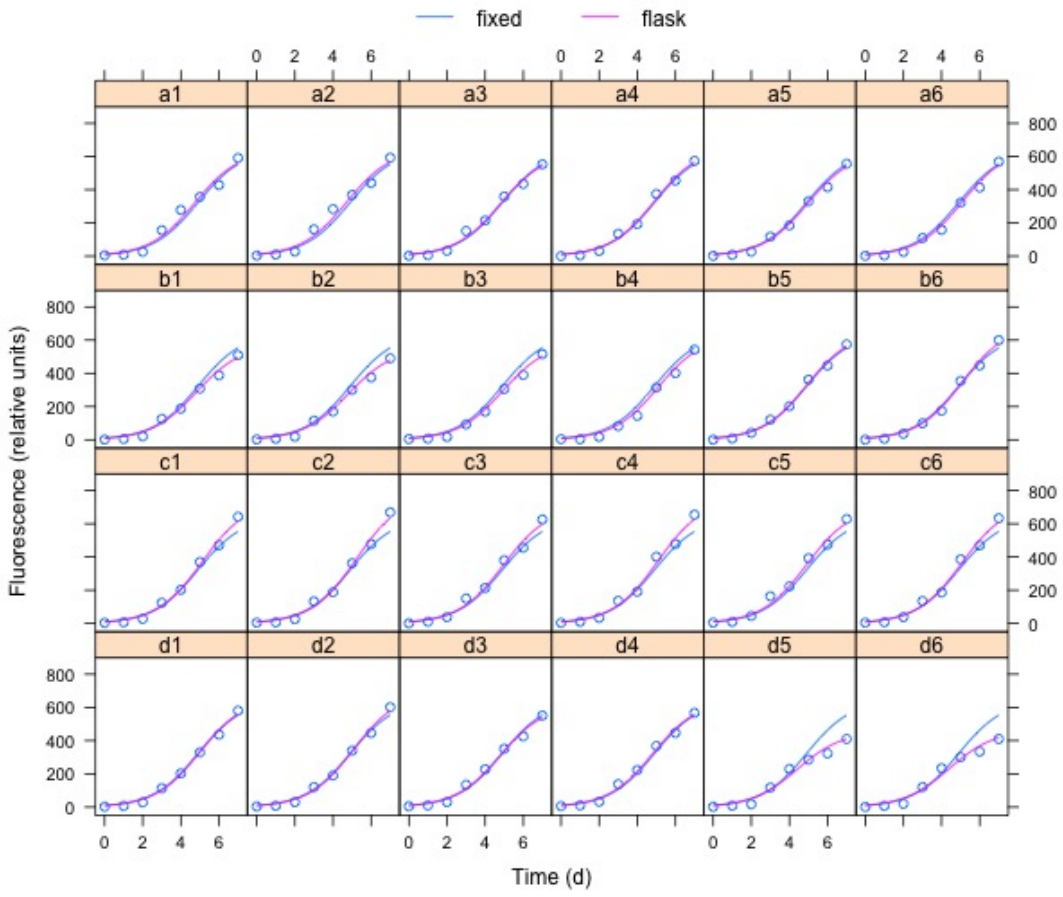


**Figure 3.12:** R plot showing fit of nlme models for *Skeletonema pseudocostatum* treated with UIO 037 filtrate. Letters a-d in each window represent each nutrient treatment (+P+N, -P-N, -P+N, +P-N). Numbers 1-6 in each window represent concentration of treatment added (100, 50, 25, 12.5, 6.25, and 0%).

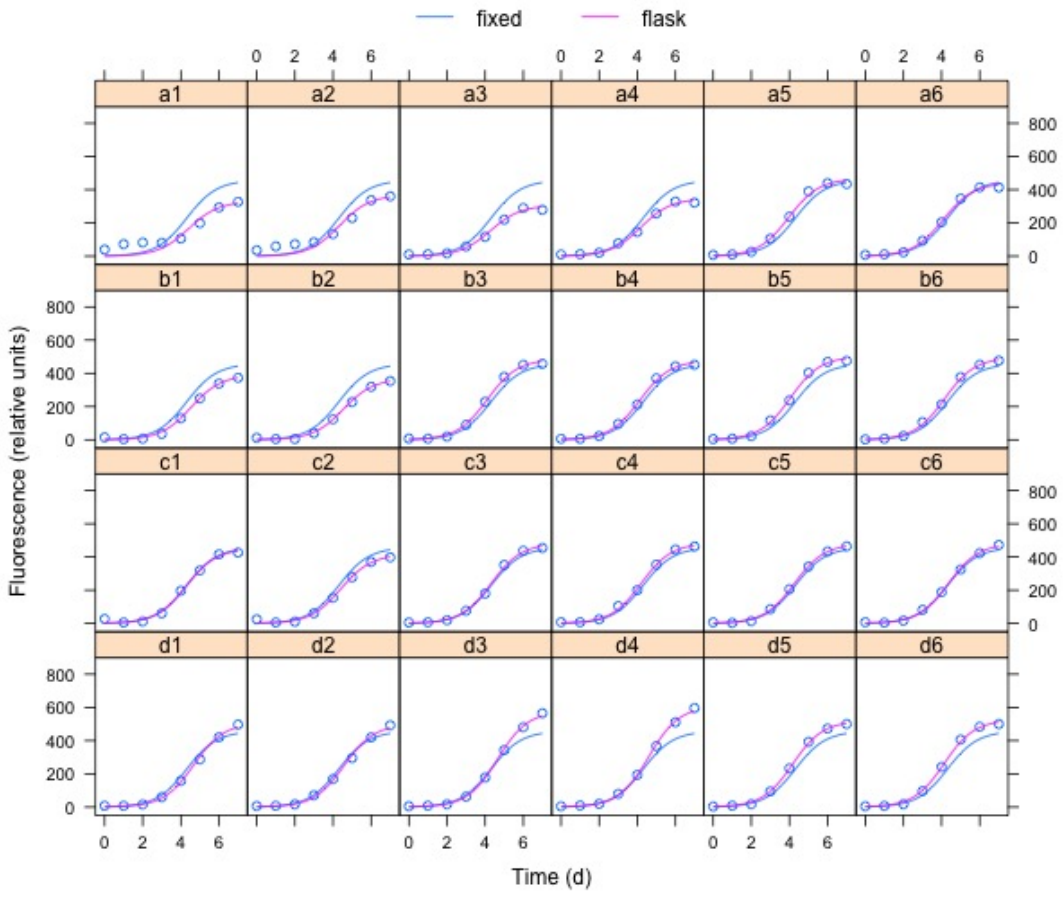


**Figure 3.13:** R plot showing fit of nlme models for *Skeletonema pseudocostatum* treated with UIO 040 lysed cells. Letters a-d in each window represent each nutrient treatment (+P+N, -P-N, -P+N, +P-N). Numbers 1-6 in each window represent concentration of treatment added (100, 50, 25, 12.5, 6.25, and 0%).

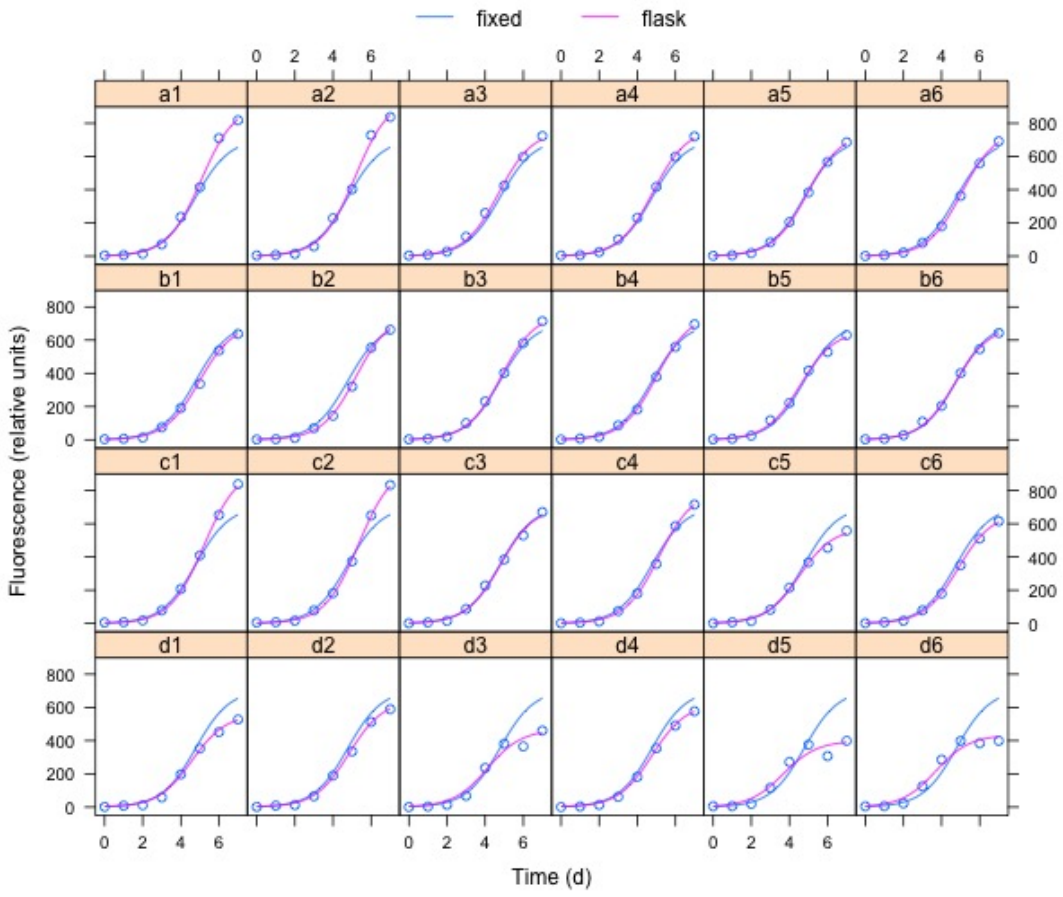


**Figure 3.14:** R plot showing fit of nlme models for *Skeletonema pseudocostatum* treated with UIO 037 filtrate. Letters a-d in each window represent each nutrient treatment (+P+N, -P-N, -P+N, +P-N). Numbers 1-6 in each window represent concentration of treatment added (100, 50, 25, 12.5, 6.25, and 0%).

*Skeletonema* cells experienced most growth inhibition under UIO 040 lysed cell treatment but due to no obvious pattern of variation between "fixed" and "flask" curves it is hard to judge if it is a significant result or not. Within treatment types, concentration does not seem to have a linear effect on growth inhibition with lower concentrations (and even in controls) sometimes showing more inhibition than at higher concentrations. Donor cells grown in +P-N (d windows in plots) seem to have a greater growth inhibitory effect but again the lack of effect of concentration makes this hard to quantify.

# Discussion

## 4.1 High throughput screening for bioactivity

Out of the eleven strains of algae screened, seven showed some activity in the apoptosis assay (UIO 004 *Micromonas pusilla*, 007 *Pseudoscourfieldia marina*, 040 *Prymnesium polylepis*, 054 *Prymnesium parvum*, 226 *Dunaliella tertiolecta*, 254 *Karlodinium micrum*, and 305 *Becheleria cincta*). In the viability assay the same seven plus one extra (UIO 015 *Synecococcus sp.*) also inhibited growth in the Jurkat cells. Extracts from UIO 040 *Prymnesium polylepis* at a dose of 50 $\mu$g/mL caused the highest levels of apoptosis out of any bioassay. In nature this species has been responsible for mass fish kills in nature as well as haemolytic activity in cod (Edvardsen, Moy, and Paasche 1990), horse (N. Johansson and E. Granéli 1999), carp (Eschbach et al. 2005), and human erythrocytes (Meldahl, Edvardsen, and Fonnum 1994) during laboratory tests. Research by the latter also assessed *P. polylepis* toxicity by tests with *Artemia salina* nauplii and the uptake of neurotransmitters Îş-aminobutyric acid and L-glutamate in rat brain cells (synaptosomes and synaptic vesicles). All four test methods indicated the haptophyte to be toxic and that this was due to the synthesis of secondary metabolites as closely related *Chrysochromulina leadbeateri* did not test positive for toxicity. These studies show that *Prymnesium polylepis* or its extracts can cause effects in organisms of both marine and terrestrial origin, supporting my findings in the first round of screening.

However, previous history of toxic blooms is not a guarantee of activity in the bioassays used here. *Prymnesium parvum* and *Karlodinium micrum* have both been responsible for ichthyotoxic blooms in the past Hallegraeff 1993; Deeds et al. 2002 but were not of note in these tests. After *P. polylepis*, the microalgae that scored highest in the apoptosis and viability assays were *Micromonas pusilla*, *Dunaliella tertiolecta*, and *Biecheleria cincta*. As far as I'm aware results showing this type of bioactivity have not been published before.

For all strains tested, ethyl acetate fractions consistently gave highest activity in the viability and apoptosis assays. For an extract to progress on to the apoptosis assay, it had to first show a certain level of activity in the viability tests. Ten of the twelve extracts

that progressed onto the apoptosis assay were extracted using ethyl acetate (the remaining two were hexane). No extracts taken with water or ethanol caused enough of a cytotoxic effect in the viability assays to be considered for the apoptosis bioassay.

### 4.1.1 Effect of growth conditions and cell life cycle stage on bioassay results

Two strains of *Prymnesium polylepis* were grown under varying nutrient conditions for the second part of this project. Using TEM, UIO 037 was deemed to be in the alternate life cycle stage while UIO 040 was authentic (according to Paasche, Edvardsen, and W Eikrem 1990) and Edvardsen and Paasche 1992). Both were tested using the same method as before but this time the aim was to discern if any variation in bioactivity was caused by life cycle stage or growth conditions. The alternate strain of *P. polylepis* caused higher inhibition of growth and cytotoxicity in the viability bioassay. Seven out of eight extracts from UIO 037 caused enough of a response to get reliable $IC_{50}$ values. For the authentic strain UIO 040 only one of eight extracts, those from cells grown at full nutrient conditions, inhibited growth to a degree that $IC_{50}$ values could be calculated. The $IC_{50}$ for strain UiO 040 was the lowest (highest cytotoxicity) of any strain tested with this method, at 0.019mg/mL. Extracts from alternate *Prymnesium polylepis* strains cells grown under phosphorous limited conditions also yielded low $IC_{50}$ values in this test (0.020mg/mL).

Previous work has also shown that culturing microalgae under nutrient limited conditions may increase toxicity. Phosphorous limited batch cultures of *Prymnesium polylepis* were found to cause higher haemolytic activity than those with sufficient nutrients (Edvardsen, Moy, and Paasche 1990; Meldahl, Edvardsen, and Fonnum 1994). In addition, it has been shown that nutrient limitation, regardless if it was P or N, leads to higher haemolytic activity (N. Johansson and E. Granéli 1999). Another study by the same authors on *Prymnesium parvum*, a close relative of *P. polylepis*, also showed increased toxicity when grown in nutrient limited conditions (N Johansson and E Granéli 1999). Phosphate limitation increased toxicity up to 20 fold in some studies of *P. parvum* (Shilo and Aschner 1953; Dafni, Ulitzur, and Shilo 1972). N and P deficient cultures of *Prorocentrum lima* and *Dinophysis acuminata* exhibited increased production of the toxin okadaic acid (McLachlan et al. 1994; Sohet et al. 1995; N Johansson, Graneli, et al. 1996). Nutrient availability seems to be central to the regulation of secondary metabolite production in phytoplankton and may be used to increase the potential for success in future bioprospecting projects. Other factors found to activate or promote toxicity in *Prymnesium spp.* are the presence of a cofactor (e.g., divalent cations and streptomycin) and of cationic polyamines, aeration (Shilo 1971) as well as pH, salinity, cell cycle and

light (Eschbach et al. 2005; Edvardsen and Paasche 1998). The use of these factors to promote bioactivity could also be of interest in future experiments.

Extracts from UIO 040 *Prymnesium polylepis* grown at sufficient nutrient concentration were the only for this strain to gain $IC_{50}$ values. At 0.019mg/mL, these were the lowest of the whole screening programme but were the only extracts from authentic cells to cause enough of a response for $IC_{50}$ to be determined. For the same nutrient conditions and extraction methods, UIO 040 consistently caused lower caspase 3/7 activity than the alternate strain UIO 037. In the past, *Artemia* toxicity tests have shown authentic *P. polylepis* to be more toxic than alternate strains (Edvardsen and Paasche 1992). This result is the opposite of my observations but the *Artemia* test utilises whole microalgae cultures while the bioassays in this masters project used fractioned extracts. It is possible that these differences in methods may be responsible for the variation in results. As *P. polylepis* cultures previously has shown different types of toxicity (haemolytic, cytotoxic, ichthyotoxic activity) it was suggested that there may be different toxins produced by this alga that cause these different effects (Edvardsen and Imai 2006). These different toxins causing different toxic effects may be produces differently in the two life cycle stages.

## 4.2 Effect of strain type and nutrient concentration on allelopathy

The aim of the allelopathy experiment was to assess the inhibition of *Skeletonema pseudocostatum* by treatments using filtrate or lysed cell cultures of Prymnesium polylepis. *S. pseudocostatum* with lowest growth rates were those treated with lysed cell cultures from the authentic UIO 040 strain. This is in conflict with the bioassay results where alternative strain UIO 037 caused most activity but agrees with previous research on the variation in toxicity of different *P. polylepis* life cycle stages using the *Artemia* test (Edvardsen and Paasche 1992). Allelopathy in *Prymnesium polylepis* has been researched on since the toxic bloom 1988. Inhibition of the activity of planktonic bacteria, ciliates and copepods by *P. polylepis* has been shown (Nielsen 1990; Tobiesen 1991) as well as direct effects on other species of microalgae (Schmidt and Hansen 2001). The latter experiment observed motility in seven dinoflagellates before and after exposure to cultures of *Prymnesium polylepis*. *Gyrodinium mikimotoi* was the only one not to lose motility and it was concluded that this may be because both target and donor species produce similar toxins. It is difficult to draw a conclusion on if growth conditions or strain type caused distinct variations but the overall result seems to agree with previous research in that Prymnesium polylepis did affect the growth of *Skeletonema pseudocostatum*.

## 4.3 Methodological aspects

While care was taken to avoid errors, reviewing the methods in the writing of this manuscript has highlighted some possible issues in the methods used. All 900mL cultures were grown in replicates of two. While all subsamples taken from these cultures were in triplicate, it would also be recommended to add a third replicate during culturing to help assess and isolate sources of variation in measurements and limit the effect of random variation on results found. In the allelopathy experiment, it is possible that lysed cell treatments from Prymnesium polylepis still contained chlorophyll that underwent fluorescence in addition to the signal generated by *Skeletonema pseudocostatum*. Measuring fluorescence in samples of lysed cell culture alone could confirm or deny if this had an effect on results. However, on average the fluorescence with *S. pseudocostatum* alone (control) did have the same level of fluorescence as the lysed cell samples at the first measurement on day 0.

## 4.4 Concluding remarks and future perspectives

The high throughput screening in this study did uncover microalgae that have not been previously shown to cause bioactivity in cultured cells. These results need to be expanded upon to investigate if the tested microalgae or their extracts can be used in new future drugs. Further screening with more Norwegian microalgae in the future could also yield new sources of metabolites for future drugs. Since nutrient conditions have been shown to affect bioactivity in this and other studies its would be of interest to investigate these variability further also.

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# Appendix A

**IMR ½ - algal medium**

(Eppley et al. 1967, modified by E. Paasche, University of Oslo).Without silicate and with selenite.

Stock solutions (made with distilled water or MilliQ-water):

Nitrate: 5 g $KNO_3$ to 100 ml

Phosphate: 0.68 g $KH_2PO_4$ to 100 ml

Trace metal solution: 6 g $Na_2EDTA$ to 1 litre,
1 g $FeCl_3 \cdot 6\ H_2O$
620 mg $MnSO_4 \cdot H_2O$
250 mg $ZnSO_4 \cdot 7\ H_2O$
130 mg $Na_2MoO_4 \cdot 2\ H_2O$
4 mg $CoCl_2 \cdot 6\ H_2O$
4 mg $CuSO_4 \cdot 5\ H_2O$
To avoid precipitation (due to $H_4EDTA$) adjust this solution to pH 8 with concentrated NaOH.

Vitamin solution: 10 mg tiamine (B-1)
0.1 mg cyanocobalamine (B-12)
0.1 mg biotine
to 100 ml MilliQ water
The two latter is added from a more concentrated stock solution due to the small amounts. This solution should be kept in a plastic bottle, preferably at –20°C.

Selenite solution: 2.63 mg $Na_2SeO_3 \cdot 5\ H_2O$ to 1 litre.

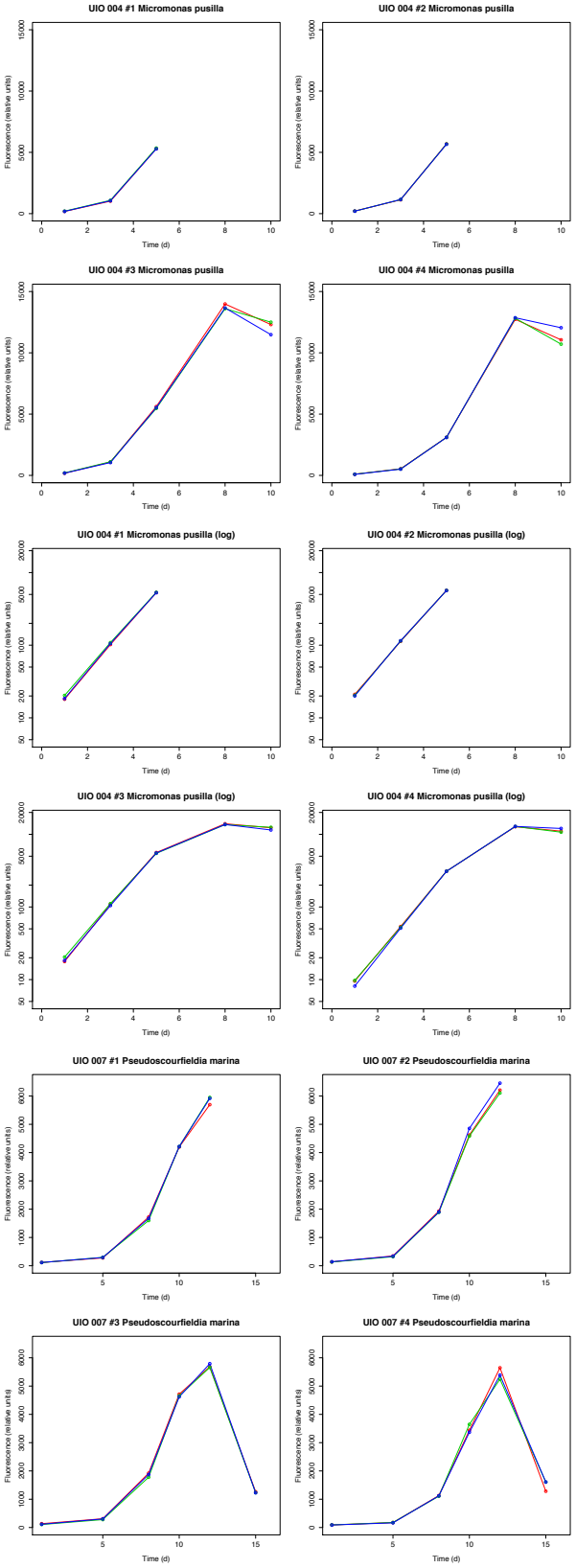
Content in 1L medium of 24‰ salinity (up to 90% seawater is fine):

| | | |
|---|---|---|
| Seawater (34 ‰ S) | 700 ml | |
| Distilled water | 300 ml | |
| Nitrate stock solution | 0.5 ml | (final concentration 250 µM) |
| Phosphate stock solution | 0.5 ml | (final concentration 25 µM) |
| Trace metal-EDTA stock solution | 0.5 ml | |
| Vitamin stock solution | 0.5 ml | |
| Selenite stock solution | 1.0 ml | (final concentration10 nM) |

All stock solutions are as described by Eppley et al. 1967, J. Exp. Mar. Biol. Ecol. 1: 191-208. The stock solutions are prepared with Milli-Q water, they are not autoclaved, and kept in the fridge. The vitamin solution can be prepared from stronger stocks that are kept in the freezer on plastic flasks. We use half the amounts of stock solutions compared to Eppley et al. (0.5 mL per liter instead of 1 mL per litre medium) and call the medium IMR 1/2. For flagellates we drop Si (and HCl) and add Se.

The seawater is filtrated (Whatman GF/C) and the medium is autoclaved for 15 min. at 120°C (110°C is probably sufficient and can be used if you get precipitation at 120°C). If distilled water is exchanged with Milli-Q-water make sure that the filter is old and rinsed from the formalin in the new filter.

# Appendix B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UIO 004 #3 Micromonas pusilla
UIO 004 #4 Micromonas pusilla
UIO 004 #1 Micromonas pusilla (log)
UIO 004 #2 Micromonas pusilla (log)
UIO 004 #3 Micromonas pusilla (log)
UIO 004 #4 Micromonas pusilla (log)
UIO 007 #1 Pseudoscourfieldia marina
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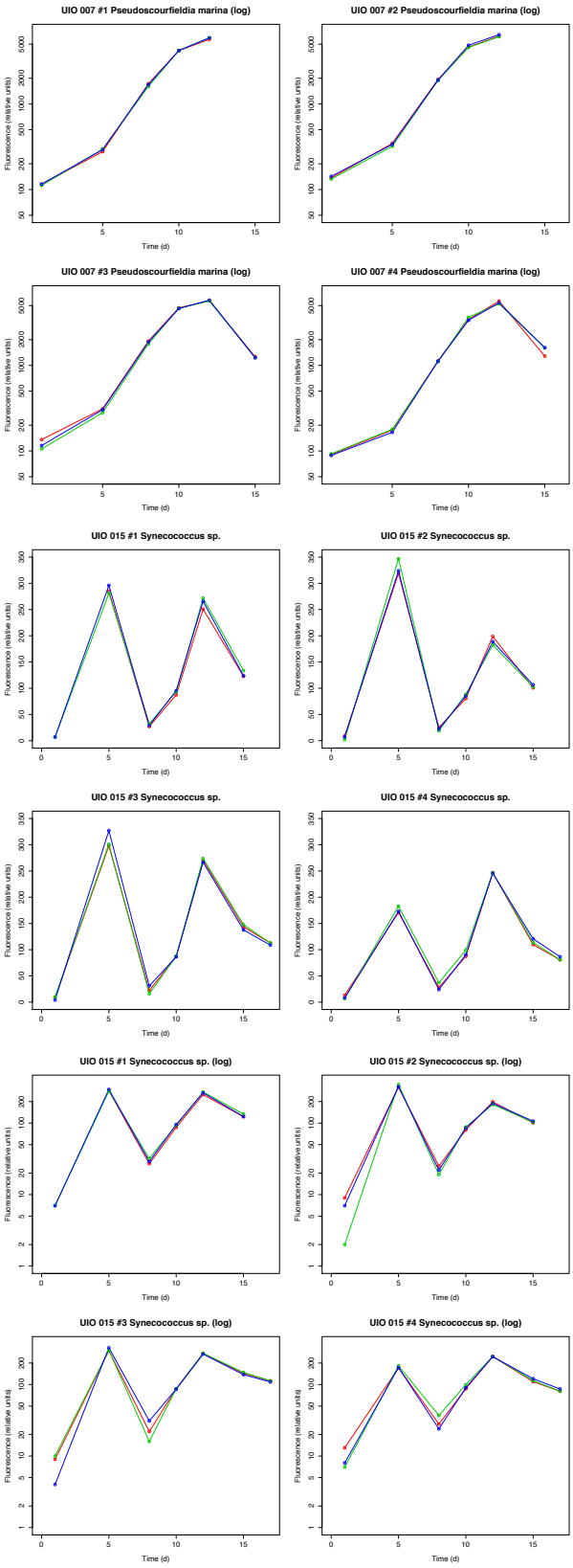
UIO 007 #3 Pseudoscourfieldia marina
UIO 007 #4 Pseudoscourfieldia marina
Fluorescence (relative units)
Time (d)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UIO 007 #2 Pseudoscourfieldia marina (log)
UIO 007 #3 Pseudoscourfieldia marina (log)
UIO 007 #4 Pseudoscourfieldia marina (log)
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UIO 015 #2 Synecococcus sp.
UIO 015 #3 Synecococcus sp.
UIO 015 #4 Synecococcus sp.
UIO 015 #1 Synecococcus sp. (log)
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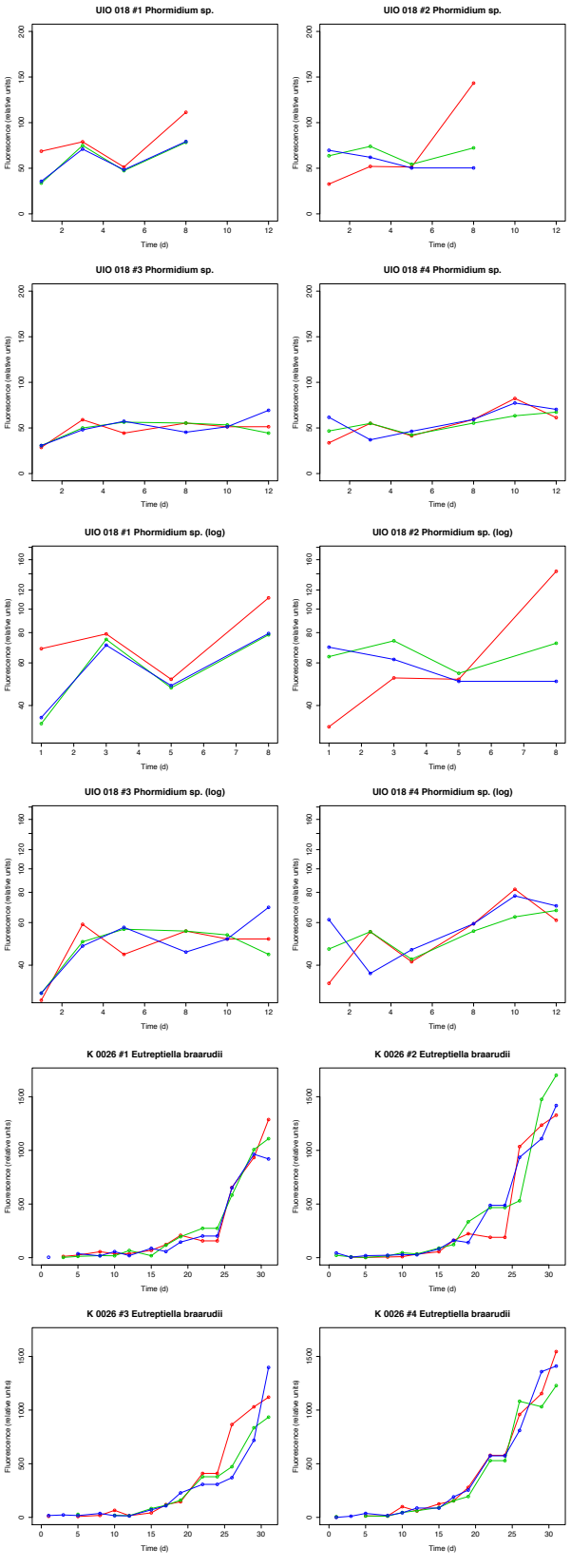
UIO 015 #4 Synecococcus sp. (log)
Fluorescence (relative units)
Time (d)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UIO 018 #3 Phormidium sp.
UIO 018 #4 Phormidium sp.
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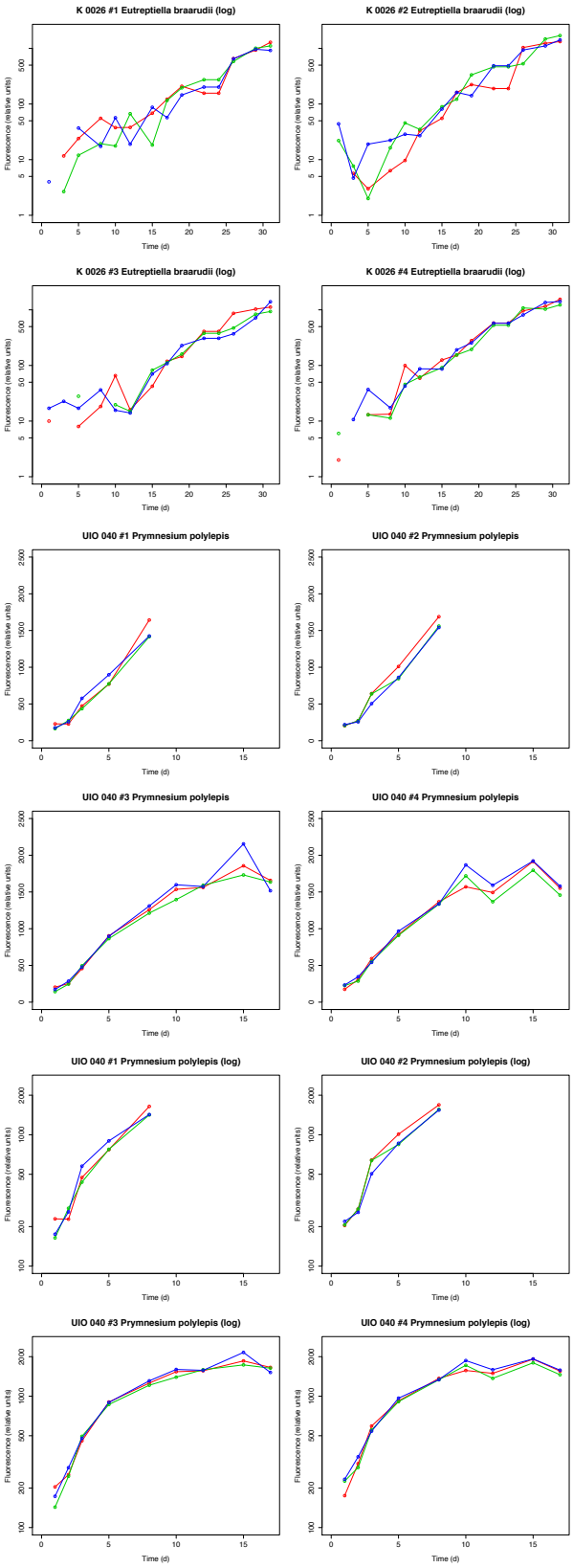
UIO 018 #2 Phormidium sp. (log)
UIO 018 #3 Phormidium sp. (log)
UIO 018 #4 Phormidium sp. (log)
K 0026 #1 Eutreptiella braarudii
K 0026 #2 Eutreptiella braarudii
K 0026 #3 Eutreptiella braarudii
K 0026 #4 Eutreptiella braarudii
Fluorescence (relative units)
Time (d)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K 0026 #3 Eutreptiella braarudii (log)
K 0026 #4 Eutreptiella braarudii (log)
UIO 040 #1 Prymnesium polylepis
UIO 040 #2 Prymnesium polylepis
UIO 040 #3 Prymnesium polylepis
UIO 040 #4 Prymnesium polylepis
UIO 040 #1 Prymnesium polylepis (log)
UIO 040 #2 Prymnesium polylepis (log)
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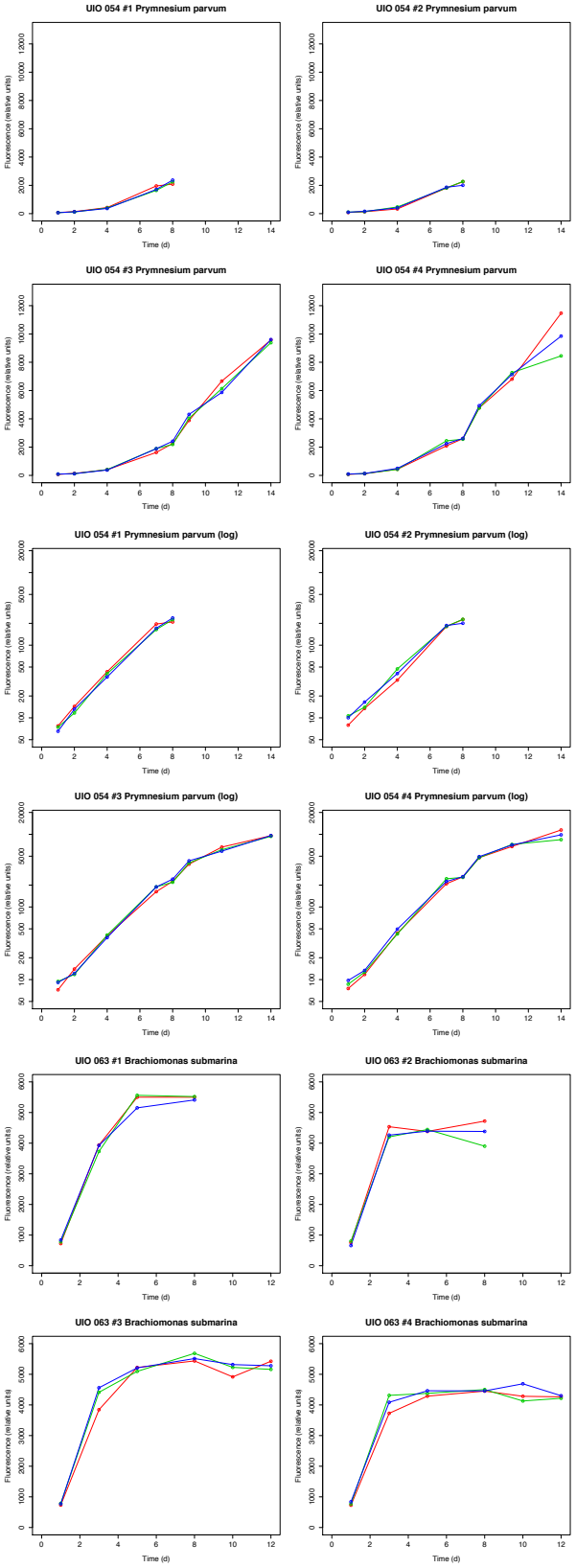
Fluorescence (relative units)
Time (d)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UIO 054 #3 Prymnesium parvum
UIO 054 #4 Prymnesium parvum
UIO 054 #1 Prymnesium parvum (log)
UIO 054 #2 Prymnesium parvum (log)
UIO 054 #3 Prymnesium parvum (log)
UIO 054 #4 Prymnesium parvum (log)
UIO 063 #1 Brachiomonas submarina
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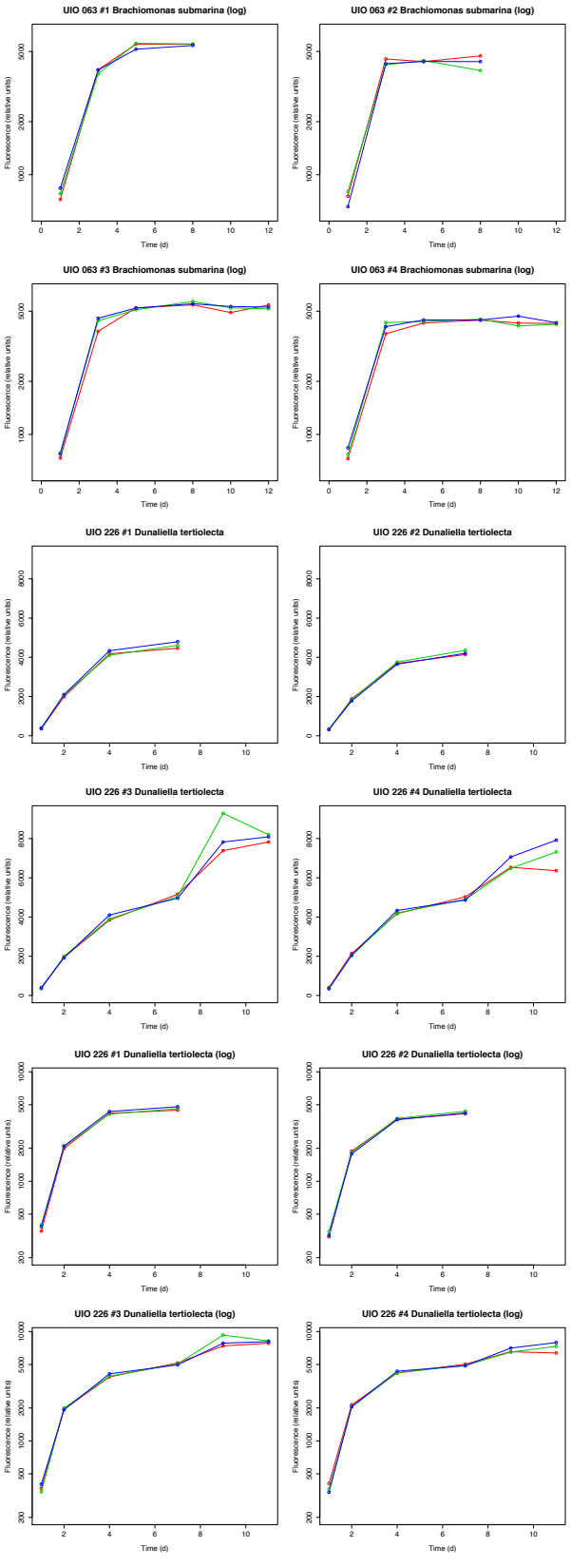
UIO 063 #4 Brachiomonas submarina
Fluorescence (relative units)
Time (d)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UIO 063 #4 Brachiomonas submarina (log)
UIO 226 #1 Dunaliella tertiolecta
UIO 226 #2 Dunaliella tertiolecta
UIO 226 #3 Dunaliella tertiolecta
UIO 226 #4 Dunaliella tertiolecta
UIO 226 #1 Dunaliella tertiolecta (log)
UIO 226 #2 Dunaliella tertiolecta (log)
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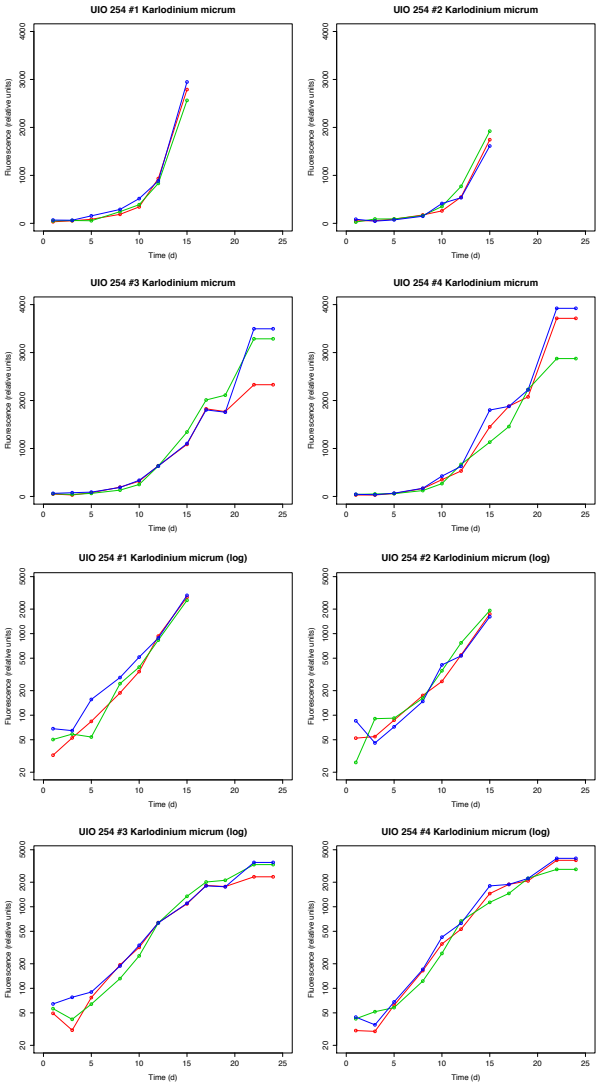
Fluorescence (relative units)
Time (d)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UIO 254 #2 Karlodinium micrum
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UIO 254 #2 Karlodinium micrum (log)
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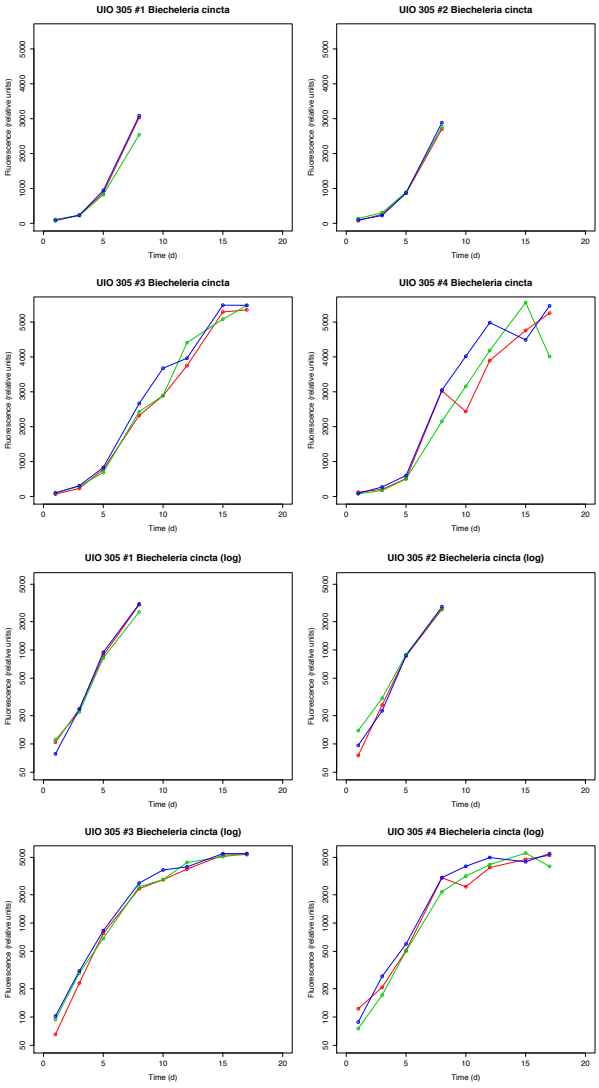
Fluorescence (relative units)
Time (d)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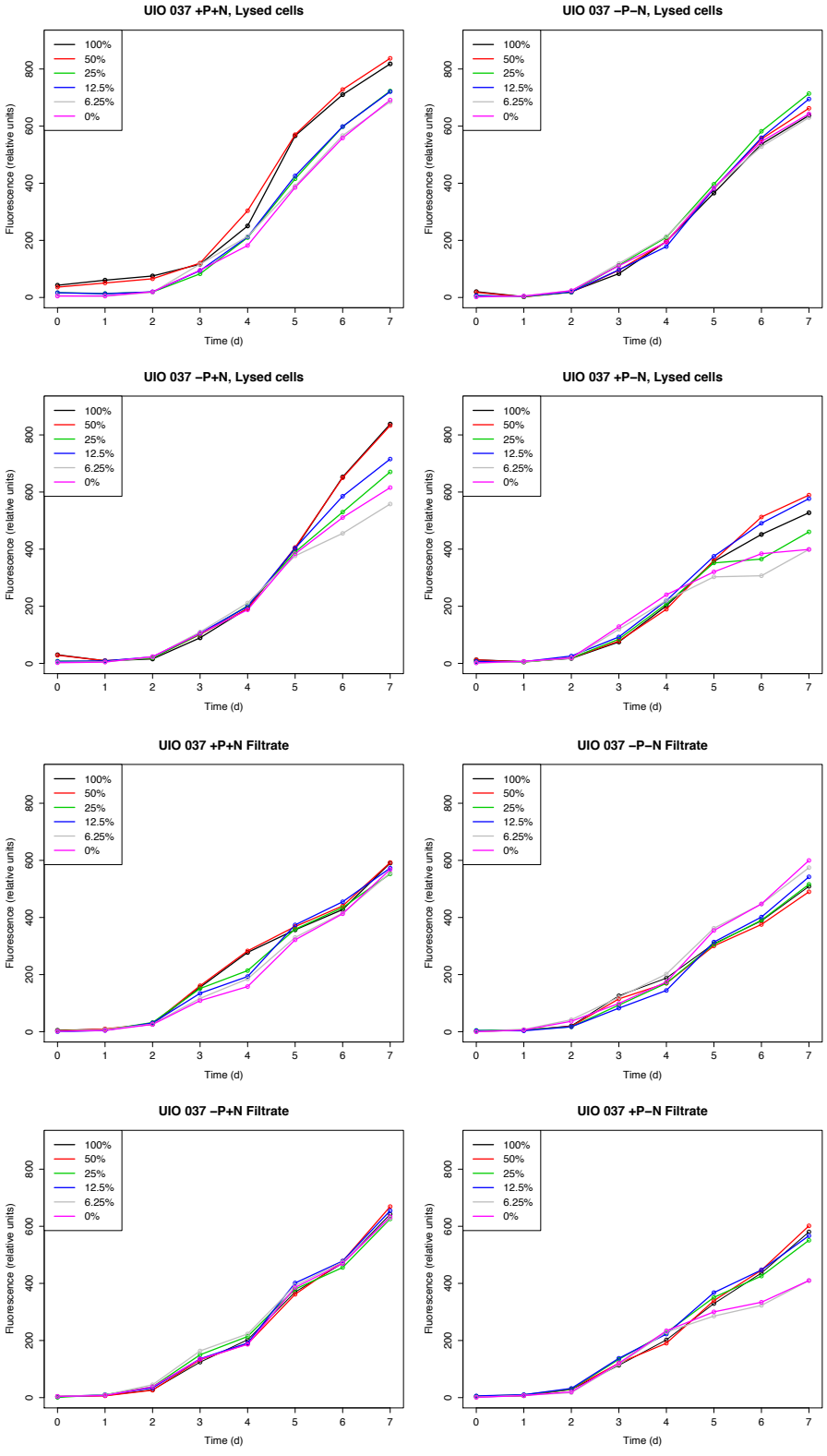
UIO 305 #2 Biecheleria cincta (log)
UIO 305 #3 Biecheleria cincta (log)
UIO 305 #4 Biecheleria cincta (log)
Fluorescence (relative units)
Time (d)

# Appendix C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UIO 037 +P+N Filtrate
UIO 037 –P–N Filtrate
UIO 037 –P+N Filtrate
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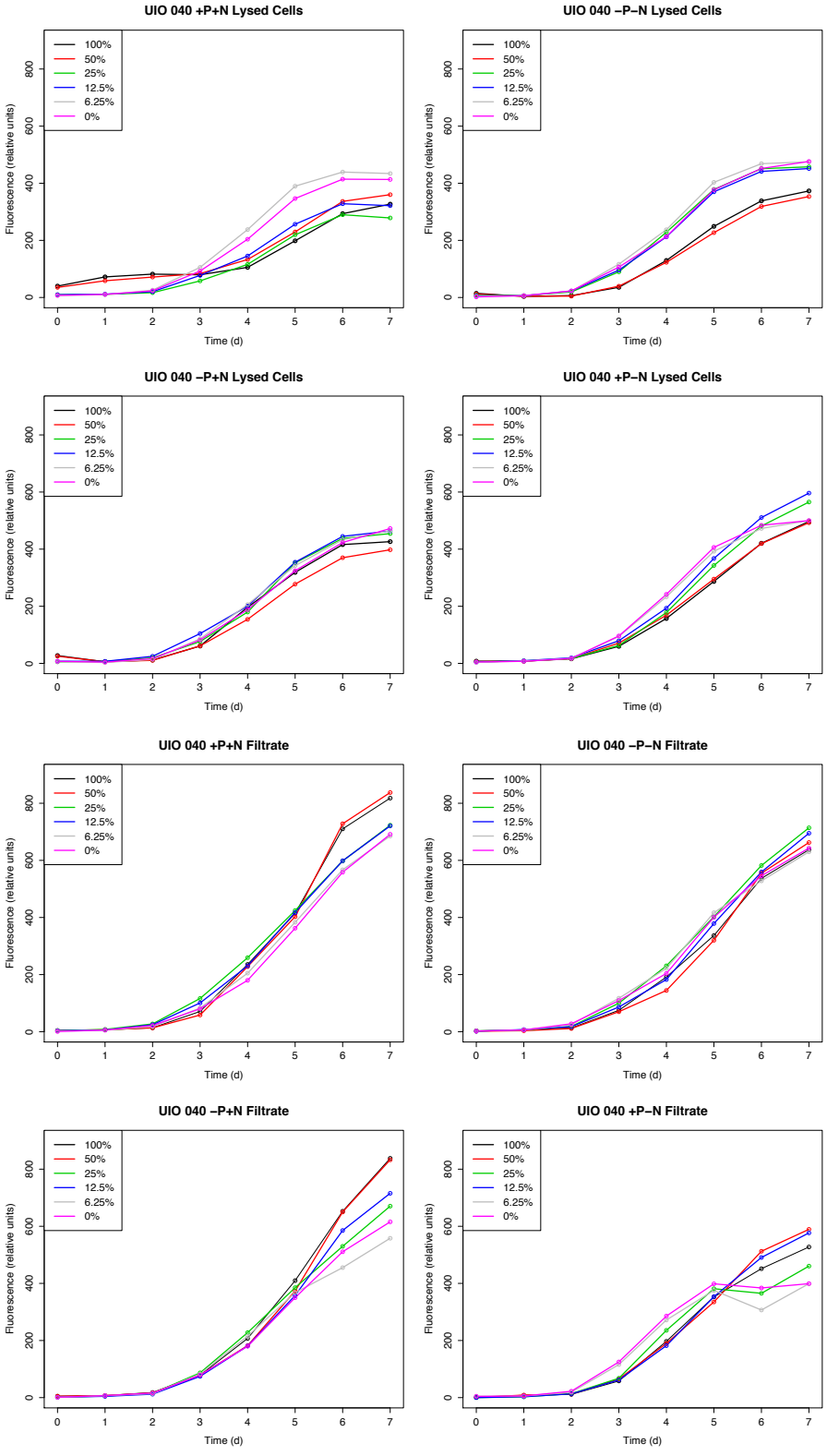
100%
50%
25%
12.5%
6.25%
0%
Fluorescence (relative units)
Time (d)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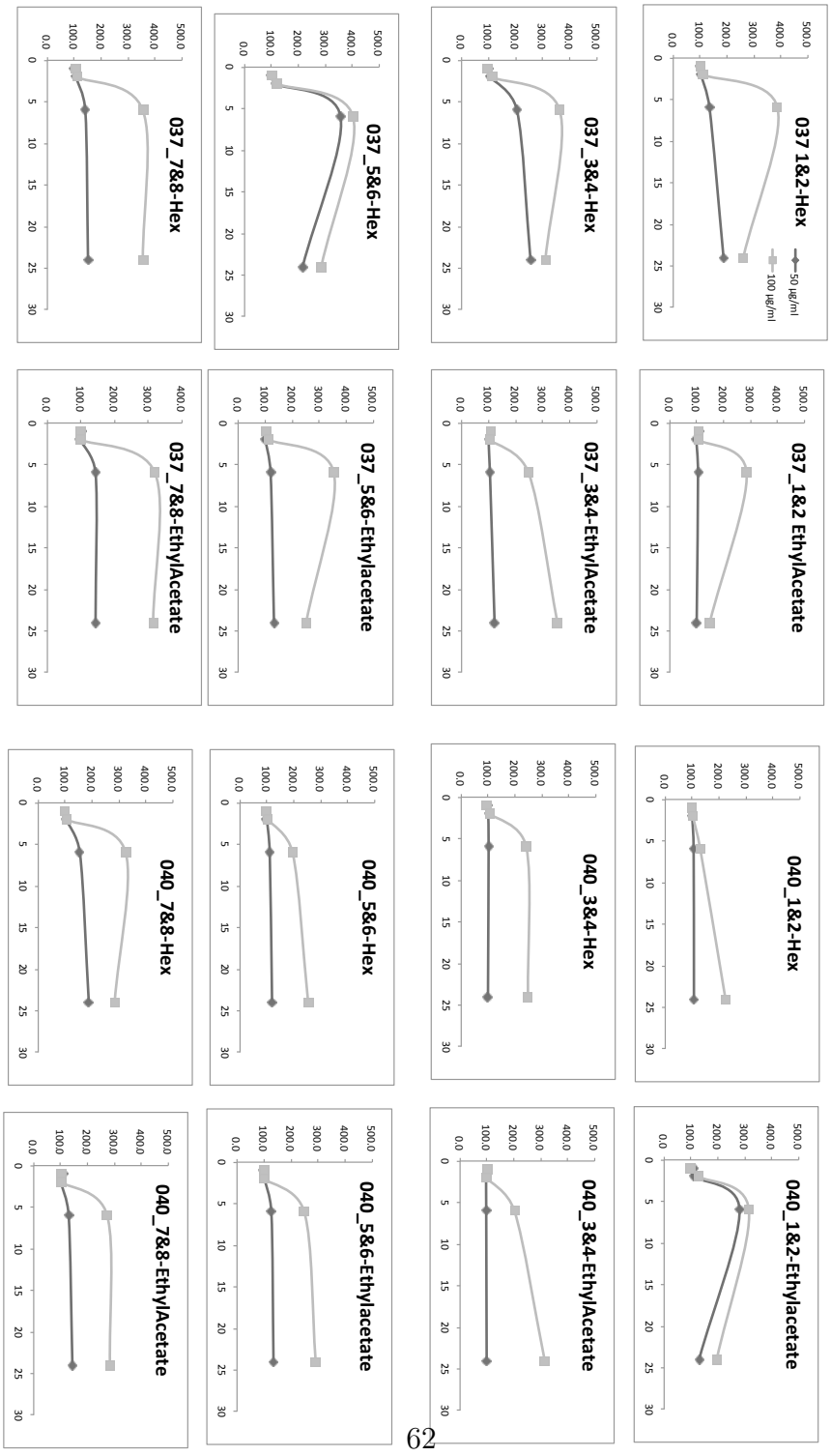
UIO 040 −P−N Filtrate
UIO 040 −P+N Filtrate
UIO 040 +P−N Filtrate
100%
50%
25%
12.5%
6.25%
0%
Fluorescence (relative units)
Time (d)

# Appendix D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